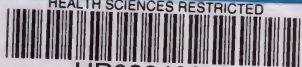


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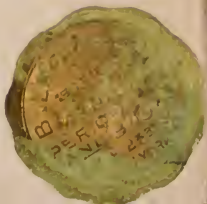
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THE
JOURNAL OF INFECTIOUS DISEASES

The
Journal of Infectious Diseases

Founded by the Memorial Institute for Infectious Diseases

EDITED BY

LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

FRANK BILLINGS F. G. NOVY

W. T. SEDGWICK

Volume 3
1906

Chicago, 1906

TABLE OF CONTENTS

No. 1, MARCH 2, 1906

	PAGES
The Bacteriology of Whooping-Cough	
<i>David J. Davis</i> - - - - -	1-36
Studies on Human Parasites in North America. I. <i>Filaria Loa</i>	
<i>Henry B. Ward</i> - - - - -	37-90
Disseminated Blastomycosis: Report of a Case Involving the Lungs, Lumbar Vertebræ, and Subcutaneous Tissues; with Multiple Abscesses and Fistulæ, and Extensive Amloid Degeneration	
<i>Peter Bassoe</i> - - - - -	91-101
The Rôle of Phagocytosis in the Anthracidal Action of Dog Blood	
<i>Ludvig Hekoten</i> - - - - -	102-109
The Anthracidal Substance in the Serum of White Rats	
<i>Jessie M. Horton</i> - - - - -	110-115
The Adjuvant Action of Serum, Egg-Albumin, and Broth on Tetanus Intoxication	
<i>H. T. Ricketts, and E. J. Kirk</i> - - - - -	116-127
Further Studies on Virulent Pseudodiphtheria Bacilli	
<i>Alice Hamilton and Jessie M. Horton</i> - - - - -	128-147
The Identity of Fusiform Bacilli and Spirilla	
<i>Ruth Tunnicliff</i> - - - - -	148-155
Further Studies on Streptococcus Infection	
<i>Gustav Ruediger</i> - - - - -	156-164
The Etiology of Pemphigus Contagiosus in the Tropics	
<i>Moses E. Clegg and William B. Wherry</i> - - - - -	165-171

No. 2, APRIL 6, 1906

The Significance of Streptococci in Milk	
<i>Paul G. Heinemann</i> - - - - -	173-182

	PAGES
A Method of Isolating Pneumococcus in Mixed Cultures, Such as Throat Cultures <i>Gustav F. Ruediger</i> - - - - -	183-186
Notes on the Common Mosquitoes of Bihé and Bailundo Districts, Portuguese West Africa <i>Fredk. Creighton Wellman</i> - - - - -	187-190
The Toxins and Antitoxins of Poisonous Mushrooms (<i>Amanita Phalloides</i>) <i>William W. Ford</i> - - - - -	191-224
On Auxilysins <i>Wilfred H. Manwaring</i> - - - - -	225-227
A New Species of Trypanosome Occurring in the Mouse <i>Mus Musculus</i> <i>Arthur I. Kendall</i> - - - - -	228-231

No. 3, MAY 18, 1906

Two Cases of Relapsing Fever with Notes on the Occurrence of This Disease throughout the World at the Present Day <i>Robert J. Carlisle</i> - - - - -	233-265
Study of a Spirochete Obtained from a Case of Relapsing Fever in Man with Notes on Morphology, Animal Reactions, and Attempts at Cultivation <i>Charles Norris, Alvin M. Pappenheimer, and Thomas Flournoy</i>	266-290
Studies in <i>Spirillum Obermeieri</i> and Related Organisms <i>Frederick G. Novy and R. E. Knapp</i> - - - - -	291-393
The Trypanosomes of Tsetse Flies <i>Frederick G. Novy</i> - - - - -	394-411
Methylene Violet and Methylene Azure <i>Ward J. MacNeal</i> - - - - -	412-433
Are Opsonins Distinct from Other Antibodies? <i>Ludvig Hektoen</i> - - - - -	434-440
Some Observations on Phagocytosis of Diphtheria Bacilli <i>H. A. Reque</i> - - - - -	441-445

TABLE OF CONTENTS

vii

	PAGES
Latent Pneumococccemia	
<i>Herman Wolf</i> - - - - -	446-451
The Etiology and Diagnosis of Hydrophobia	
<i>Anna Wessels Williams and May Murray Lowden</i> - - -	452-483

No. 4, JUNE 30, 1906

A Statistical Study of Generic Characters in the Coccaceae	
<i>C. E. A. Winslow and Anne F. Rogers</i> - - - - -	485-546
The Toxic Effect of Certain Acids upon Typhoid and Colon Bacilli in Relation to the Degree of Their Dissociation	
<i>C. E. A. Winslow and E. E. Lochridge</i> - - - - -	547-571
A Contribution to the General Principles of the Pharmacodynamics of Salts and Drugs	
<i>A. P. Mathews</i> - - - - -	572-609
Studies on Simple and Differential Methods of Staining Encapsulated Pneumococci in Smear and Section	
<i>Augustus Wadsworth</i> - - - - -	610-618
Liver Necrosis and Venous Thrombosis in Horses Actively Immunized with Diphtheria and Tetanus Toxins and with Streptococci and and Their Products	
<i>Herbert D. Pease and Richard M. Pearce</i> - - - - -	619-637
A Fundamental Error in Current Attempts to Apply Physical Chem- istry to Serum Pathology	
<i>Wilfred H. Manwaring</i> - - - - -	638-646
The Third Serum Component	
<i>Wilfred H. Manwaring</i> - - - - -	647-662
The Cause of Green Coloration of Bacterial Colonies in Blood-Agar Plates	
<i>Gustav F. Ruediger</i> - - - - -	663-665
Generalized Blastomycosis	
<i>E. E. Irons and E. A. Graham</i> - - - - -	666-682
The Rôle of Phagocytosis in the Pneumococcal Action of Pneumonic Blood	
<i>Edward C. Rosenow</i> - - - - -	683-700

No. 5, OCTOBER 30, 1906

	PAGES
The Oral Administration of Antitoxins for Prevention of Diphtheria, Tetanus, and Possibly Sepsis, with Some Observations on the Influence of Certain Drugs in Preventing Digestion and Promoting Absorption from the Alimentary Canal	
<i>Charles T. McClintock and Walter E. King</i> - - - - -	701-720
Phagocytosis of Red Corpuscles	
<i>Ludvig Hektoen</i> - - - - -	721-730
Observations on the Opsonic Index and the Antipneumococcal Power of the Blood in Pneumonia	
<i>Herman E. Wolf</i> - - - - -	731-741
Concerning the Production of Cytotoxic Sera by the Injection of Nucleo-proteids	
<i>Richard M. Pearce and Holmes C. Jackson</i> - - - - -	742-754
The Streptococci from Scarlatinal and Normal Throats and from Other Sources	
<i>Gustav F. Ruediger</i> - - - - -	655-769
A New Simple Method for Staining the Polar Bodies of Diphtheria Bacilli	
<i>Albert A. Epstein</i> - - - - -	772-773
A Simple Method of Sterilizing Blood for Cultural Purposes	
<i>Eugene P. Bernstein and Albert A. Epstein</i> - - - - -	772-773
Mouth Disinfection in the Prophylaxis and Treatment of Pneumonia	
<i>Augustus Wadsworth,</i> - - - - -	774-797

The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 3

March 2, 1906

No. 1

THE BACTERIOLOGY OF WHOOPING-COUGH.*

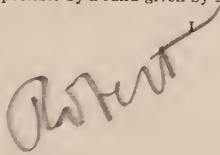
DAVID J. DAVIS.

(From the Memorial Institute for Infectious Diseases, Chicago.)

CONTENTS.

- I. INTRODUCTION.
 - Importance of Determining the Cause of Pertussis.
 - Reasons for Assuming it an Infectious Disease.
 - Possible Nature of the Virus.
- II. LITERATURE.
 - Protozoa.
 - Cocci.
 - Bacilli.
 - Conclusions from Literature.
- III. PERSONAL OBSERVATIONS.
 - Technique and Material.
 - Bacteriology of Cases.
 - Influenza-like Organisms.
 - Morphology.
 - Cultural Characteristics.
 - Symbiosis.
- IV. ANIMAL EXPERIMENTS.
 - Literature.
 - Experiments upon Lower Animals.
 - Human Inoculation.
- V. INFLUENZA-LIKE ORGANISMS IN OTHER DISEASES AND IN NORMAL THROATS.
 - Measles. Influenza. Meningitis. Bronchitis. Varicella. Normal Throats.
 - Literature.
 - Pertussoid.
- VI. NOMENCLATURE OF THE BACILLI.
- VII. SUMMARY AND CONCLUSIONS.

*This work was made possible by a fund given by Mrs. F. R. Lillie for the study of whooping-cough.



I. INTRODUCTION.

THE etiology of whooping-cough has not as yet been determined, notwithstanding the large number of investigations upon this subject. The disease has many serious aspects, and the problem of its etiology surely deserves to be ranked among the most important of the still numerous problems concerning infectious diseases that so far have not been solved. Like many other infectious diseases, the therapeutics of whooping-cough is in a most unsatisfactory condition; and it is likely to remain so, at least until the etiology is determined. No specific is known, and while some drugs may mitigate the symptoms slightly, none has any appreciable effect upon the general course of the disease. As regards its prevalence, it occupies a position in the first ranks of the infectious diseases of children, probably being equaled only by measles in this respect. While in strong, healthy, and older children the disease may not be considered very serious, however, owing to its prolonged course, especially in the winter months, with an abundance of serious complications and sequelæ, it must be looked upon, as the high rate of mortality abundantly proves, as one of the most dreaded diseases for the young infants and the more delicate children.

Reasons for assuming it an infectious disease.—There can be no doubt that whooping-cough is an infectious, transmissible disease. Several convincing reasons may be given for this. Transmission of the disease certainly occurs by contact between children, and Baginsky states positively that he has observed transmission by a third person. There is no doubt that the virus adheres to rooms and fomites. The frequent epidemic character of the disease, and its endemic character in cities, indicate its infectious nature. Again, the disease has a fairly definite period of incubation. There is some difference of opinion as to the length of this period, but most observers agree that it is from 7 to 14 days. Immunity is as a rule conferred by the attack. Indicative of its infectious nature is also the relatively high leucocytosis, which varies from 12,000 to 45,000, the increase in the mononuclears being particularly characteristic. Not only does the clinical course of the disease indicate an infectious catarrhal condition of the upper respiratory passages, but the pathologic anatomy substantiates such a conclusion. The mucosa, especially of the larynx and trachea,

is hyperemic and usually covered with more or less mucus. According to Arnheim, who has had numerous postmortems, there is a marked desquamative catarrh of the larynx and trachea, with swelling of the neighboring lymph glands. We know also that the posterior laryngeal wall, near the vocal cords, is the point whence severe coughing may be most readily produced, and this locality is supplied by the superior laryngeal nerve, the one which produces cough when irritated. This may be given as a reason for viewing the disease as an inflammatory irritation of the laryngeal mucosa; however, the possibility of reflex coughs due to irritation of the nasal mucosa, and the possibility of a toxic irritation of the coughing-center in the medulla, though speculative in character, might be considered.

The above facts, therefore, furnish strong evidence that the disease is due to some form of a zymotic agent, localized in all probability in the upper respiratory passages, particularly the larynx and trachea. This is important in determining the point of attack in attempting to solve the problem of etiology.

Possible nature of the virus.—From what is known concerning the nature of the causative virus of diseases of an infectious character, we may consider three possible agents, viz.: protozoa, bacteria, and enzymes. The last-mentioned agent may be dismissed by saying that, in the animal world at least, there does not exist at present evidence to indicate that such substances play any part whatever in the primary causation of any known infectious diseases. In the plant world, however, certain recently discovered facts seem to point to the possibility of enzymes being the cause of certain diseases of an infectious nature; and if this be true we should guard against overlooking such a cause in studying animal diseases of unknown etiology. Protozoa and bacteria are the common agents in the production of disease, and it is among these that we naturally look for the final solution of the problem. They may be microscopic or ultramicroscopic. We are familiar today with several diseases in animals which are undoubtedly due to ultramicroscopic organisms. Such are foot-and-mouth disease, hydrophobia, and probably hog-cholera. It has been demonstrated that these can be produced in animals, and therefore abundant material is available for careful experimental work. Whooping-cough, so far as we know, does not occur, and cannot be

produced, in animals, and we are therefore handicapped greatly in not being able to apply this method to the study of the disease. There is little doubt but that its cause, as well as that of many other human diseases of unknown origin, would be soon cleared up if only available material for experimentation could be procured. The possibility, however, of an etiologic ultramicroscopic organism should be kept in mind in studying this disease, and methods devised accordingly.

II. LITERATURE.

The literature upon the bacteriology of pertussis is as confusing as it is extensive. Almost every year one or more new organisms are described and assigned an etiologic rôle in the disease. Curiously enough, many of the investigators find organisms differing from those found by every other investigator, and their enthusiasm has not infrequently led them into controversies of a more or less personal character. After going over the literature one is clearly impressed with the idea that a great deal of rather superficial work has been done on the subject, and very little that is really thorough.

For convenience it may be well to divide the findings, as Jochmann and Krause have done, into three classes, namely protozoa, cocci, and bacilli, and discuss each briefly.

Protozoa.—Henke¹ in 1874 and Deichler² in 1886 found constantly, in the sputum of whooping-cough patients, bodies which they interpreted as protozoa. Kurloff³ in 1896 reported similar findings. From the illustrations accompanying his paper one can scarcely draw any conclusion other than that they are desquamated, ciliated epithelial cells. Behla⁴ in 1898, who also found ameba-like bodies in fresh pertussis sputum and assigned to them an etiological significance, interprets Kurloff's ciliated bodies as epithelial cells, but says that the ameba-like bodies, which Kurloff also described, were the same as his bodies. All of the above results were inadequately controlled and only indefinite data are given concerning the cases examined. The interpretations, for the most part, have been based upon observations made in unstained specimens. Behla states that staining was of no differential diagnostic value. The usual interpretation given to these results by later investigators is that leucocytes and epithelial cells have been mistaken for protozoa.

Cocci.—Certain early observations, such as those recorded by Moncorvo, Barlow and Broadbent, Haushalter and Mircoli, are of no significance, and need not be discussed. Ritter⁵ in 1892, and later in 1896, described a small diplococcus which he obtained constantly from 146 cases of pertussis, and which he considered the probable

¹ *Deutsches Arch. f. klin. Med.*, 1874, 12, p. 630.

² *Centralbl. f. Bakt.*, 1896, 19, p. 513.

³ *Baumgarten's Jahresbericht*, 1886, 2, p. 347.

⁴ *Deutsche med. Wchnschr.*, 1898, 24, p. 299

⁵ *Berl. klin. Wchnschr.*, 1892, 29, p. 1276; also 1896, 33, p. 1040.

cause. His results could not be verified by Cohn and Neumann or by Czaplewski and Hensel. In 1899 his assistant, Buttermilch,¹ attempted to identify Ritter's organism with the organism described by Vincenzi and also with that described by Czaplewski and Hensel. Neither Ritter nor Buttermilch used blood media, so that their results are not directly comparable with those obtained by later investigators.

Bacilli.—Burger² in 1883 was probably the first to call attention to numerous bacilli in the sputum of whooping-cough patients. He made no culture experiments, studying only stained preparations. He called attention to the possibility of diagnosing the disease from the very large number of organisms seen in the sputum. His results are of little value, because no cultures were made. Afanassiew,³ in 1887, described short motile, bacilli, often in masses and chains. He claims that he was able to produce the disease in dogs and rabbits by the injection of pure cultures of the bacillus. He studied 10 cases. He did not use blood media, and his method of obtaining pure cultures is questionable. Von Genser, Wendt, and Szemetschenko claim to have substantiated his results.

Spengler⁴ was apparently the first to describe an organism, in pertussis sputum, closely resembling the influenza bacillus in its morphological and biological aspects. His organisms are somewhat thicker and larger than the influenza bacillus, and have often a tendency to thread formation. Sometimes the protoplasm of the cells is filled with them. They grow only upon hemoglobin media. He considered his organism of etiological importance in the disease.

Koplik⁵ found a small, short, motile bacillus in smears and culture in the sputum in 13 out of 16 cases investigated. It grew on ordinary media, but thrived much better when hydrocele fluid was added. It was pathogenic to white mice, and intravenous injections into rabbits caused pyemia. It is very doubtful whether he was working with pure cultures, as his method of isolating the organism was hardly reliable. His results with animals suggest the presence of some of the ordinary pus producers of the mouth and throat.

Czaplewski and Hensel⁶ likewise found a small, short, polar-staining bacillus, slightly larger than the influenza bacillus, but which grew upon non-hemoglobin media. They isolated the organism from sputum on blood-serum plates. Morphologically it resembled very closely the influenza-like organisms described by Spengler, Jochmann, and Vincenzi. Buttermilch contended that it was identical with Ritter's diplococcus. Spengler said that it was the same as his bacillus, but that Czaplewski and Hensel did not obtain it in pure culture, and hence, when transferred to other media, the mixed organisms developed which were mistaken for the bacilli. Czaplewski's results were confirmed independently by Zusch,⁷ who used very much the same technique, and also by A. Cavasse. The latter,⁸ in 1904, described another organism, motile, growing only in liquids, staining very poorly, and never before observed in pertussis sputum; its significance he does not attempt to indicate. Vincenzi,⁹ in 18 cases, found a small, influenza-like organism, non-Gram staining, which grew on non-hemoglobin media. He says that it is different from Czaplewski's organism.

Elmassian¹ described an organism identical with Pfeiffer's influenza organism,

¹ *Ibid.*, 1899, 36, p. 367.

² *Ibid.*, 1883, 9, p. 7.

³ *St. Petersburger med. Wchnschr.*, 1887, 4, p. 349.

⁴ *Deutsche med. Wchnschr.*, 1897, 23, p. 830.

⁷ *Centralbl. f. Bakt.*, 1898, 24, pp. 721, 769.

⁵ *Centralbl. f. Bakt.*, 1897, 22, p. 222.

⁸ *Arch. gén. de Méd.*, 1904, 193, p. 1345.

⁶ *Deutsche med. Wchnschr.*, 1897, 23, p. 586.

⁹ *Deutsche med. Wchnschr.*, 1898, 24, p. 631.

except that it grows on serum media. He found it in eight out of 32 cases of whooping-cough, and also obtained it from cases of acute bronchitis, pulmonary tuberculosis, and pneumonia. He thinks it very probable that it is the same as Pfeiffer's bacillus, which he likewise tested, and found that it also would grow upon his serum agar. He does not consider it proved that influenza is caused by Pfeiffer's organism. Animal experiments were for the most part negative. He questioned its etiological significance for whooping-cough. There can be little doubt that this organism is the same as that described by Spengler, and later by Jochmann and Krause.

Luzzatto's *Bacillus minutissimus sputi*² corresponds to Elmassian's bacillus in practically every respect. He studied 41 cases. He also described an organism resembling the pneumococcus.

Arnheim,³ in 1900, using Czaplewski's technique, found Czaplewski and Hensel's organism both in sputum of patients and in two out of three autopsies. In 1903 he reported his findings in eight autopsies. He claims to have cultivated the organism from the lung tissue and tracheal mucus. In sections he observed the bacteria especially in the wall of the trachea, in the lungs, and in great numbers in the large desquamated epithelial cells. The organisms grow on ordinary media, and his cultures show very irregular coccus forms, thread forms, with enlarged ends, and various involution forms. With Gram's method of staining, he says that some of the organisms stain and some do not.

Jochmann and Krause,⁴ in 1901, found influenza-like bacilli in sputum of pertussis cases which belonged to three distinct classes (A, B, C) as determined by their reaction to Gram's stain and by their biological properties. In 18 out of 31 cases, among which were three autopsies, were found small, non-Gram-staining, influenza-like bacilli (Class A), which grew only in the presence of hemoglobin. This organism they called *Bacillus pertussis* Eppendorf. In four cases they found similar bacilli (Class B), which, however, grew on hemoglobin-free media. They considered this organism the same as that described by Czaplewski and Hensel, and thought, because so infrequently found, that it could not be the cause of pertussis. In three cases they found a Gram-staining bacillus (Class C) growing without the presence of hemoglobin. The occurrence of these various forms in pertussis, they think, explains the discordant results of the previous investigations. In 1903 Jochmann⁵ reports finding the *B. pertussis* Eppendorf altogether in 60 cases and in 23 autopsies. He does not report further findings of organisms belonging to the Classes B and C. He says that one has as much right to consider this bacillus the cause of whooping-cough as to consider the influenza bacillus the cause of influenza.

In 1903, Reyher⁶ reports the constant presence of Czaplewski's "Polbacterium" in 34 cases of pertussis. He found them in sputum, once in pus from the ear, sometimes in the nasal secretion, in the mucus of the larynx and trachea of nine autopsies, and in one instance saw them in sections of the larynx and trachea in the epithelial cells. He states that the organism destains by Gram. He cannot confirm Jochmann's work. His report in many respects is very indefinite.

In 1903 Neisser,¹ from conjunctivitis in measles, obtained *B. xerosis* and an influenza organism which he cultivated together for 20 generations on plain agar. He tested strains from pertussis, scarlet fever, and measles, and could not detect any difference

¹ *Ann. de l'Inst. Past.*, 1899, 13, p. 621.

² *Centralbl. f. Bakt.*, 1900, 27, p. 817.

³ *Berl. klin. Wchnschr.*, 1900, 37, p. 703; also *Virchow Archiv*, 1903, 174, p. 530.

⁴ *Ztschr., f. Hyg.*, 1901, 36, p. 193.

⁵ *Ibid.*, 1903, 44, p. 513.

⁶ *Jahrbuch f. Kinderh.*, 1903, 58, p. 605.

morphologically, culturally, or by immunization experiments. They grew well through many generations with living *B. xerosis*, but not with the dead organisms or their extracts.

Manicatide² found a short, thick, Gram-staining bacillus capable of growing on ordinary media in 67 out of 80 cases, and says it is different from any organism previously described. Animal experiments gave negative results. He immunized three sheep and two horses, and obtained a distinct agglutination at a dilution of 1:32. He treated 89 cases with the immune serum, and claims that it cures cases not over 15 days old in from 2 to 12 days. For the above reasons, he considers this germ the specific cause of the disease.

Smit³ found, in 24 cases, Jochmann's *B. pertussis* Eppendorf constantly. Neither blood serum of patients nor the serum of a horse or goats immunized to this organism had any agglutinative properties. In rabbits, by inoculation with this organism, he obtained lameness and a general infection, and also produced a conjunctivitis with the bacilli in the exudate. He treated nine patients with immunized horse serum with no perceptible effect. In 18 cases he failed to find Manicatide's bacillus in a single instance. With the bacillus which he obtained from Manicatide, he obtained some agglutination with the blood serum of pertussis patients. He does not consider either Jochmann's bacillus or Manicatide's bacillus as the cause of the disease.

Very recently Martha Wollstein⁴ isolated from 29 out of 30 cases a bacillus identical with that described by Spengler and Jochmann. This organism was agglutinated by serum of pertussis patients at dilutions of 1:200, and sometimes at 1:500. With normal serum, the tests all gave negative results above dilutions of 1:10. With influenza bacilli, the serum of pertussis patients did not react in dilutions higher than 1:20. Serum from rabbits immunized with the pertussis organism gave positive agglutination tests with the same organism at dilutions of 1:500, with the influenza organism at 1:200. Serum from animals immunized with influenza bacilli agglutinated the influenza organism at 1:200 and the pertussis organism at 1:50. Absorption tests with immunized sera also showed marked reduction in agglutinins after a saturation with the bacilli. She concludes that the pertussis organism belongs to the influenza group, but can be differentiated from *B. influenzae* by agglutination reactions with the blood of patients and immune animals. Morphologically it is slightly larger than the influenza bacillus.

Conclusions from literature.—In summing up the literature on pertussis the most striking feature is the lack of unity in the results. The work of the earlier investigators is of little value, because bacteriological technique at that time was so little developed. Undoubtedly the chief reason for the diversity of results lies in the variety of the methods employed. Lack of knowledge of the normal flora of mouth and throat also has probably had much to do with the variety of results obtained. Many of the investigators have not at all appreciated the importance of careful control of their results. These facts, together

¹ *Deutsche med. Wchnschr.*, 1903, 29, p. 462.

² *Ztschr. f. Hyg.*, 1904, 45, p. 469.

³ *Bacteriologische Onderzoekingen bij Kinkhoest*, Thesis, Amsterdam, 1905.

⁴ *Jour. of Exper. Med.*, 1905, 7, p. 335

with an evident attempt on the part of some of the investigators to exaggerate minor differences in order to be able to announce their organism as "heretofore undescribed," have been responsible for the resulting chaotic mass of data.

However, there has been found with some degree of constancy, in the sputum of patients, a small, short bacillus, often described as influenza-like, both in smears and in culture; and it may be further stated that, with few exceptions, it has been a Gram-negative organism. Further than this one finds little harmony in the results. The organism described by Spengler and by Jochmann and Krause grew only on hemoglobin media. Those described by Luzzatto and Elmassian grew in human serous fluids, and as these fluids often contain small amounts of hemoglobin, these latter organisms are probably the same as those described by Spengler and by Jochmann and Krause. Jochmann considers his organism different from that described by Spengler because of certain minor morphological differences. There is no doubt but that both are dealing with the same organism, as Spengler has very emphatically contended. The later findings of Smit and Wollstein strongly emphasize the significance of this organism. Just what organism Czaplewski and Hensel, Arnheim, Reyher, and some others, have been dealing with is very difficult to say. After reading their articles one is inclined to question, as Spengler did, whether or not they were dealing with a pure culture. Their varying reports concerning its reaction to Gram's stain, its morphology (Arnheim), and the questionable methods used in obtaining pure cultures, make one suspicious of the whole work. It is probable that they have been dealing with the same organism as that which Spengler and Jochmann described. This organism will grow, for a few generations at least, on non-hemoglobin media if another organism is present with which it may grow in symbiosis. Therefore, if sputum is smeared on such media as Czaplewski used, the Spengler-Jochmann organism will grow because there are always other organisms present. But it is a difficult matter to obtain it in pure culture, and Czaplewski and Reyher speak of the trouble they had in this respect.

No one has found an organism, either before or since Manicatide's report, that corresponds to that described by him. This is all the

more remarkable, since his bacillus grows readily on all ordinary media. He very kindly sent me a culture of his organism, which I was thus able to compare with the various organisms found in pertussis sputa of the cases reported in this paper, and in not one instance have I been able to obtain an identical organism. It is an entirely different organism from the influenza-like bacillus.

Summing up, one seems justified in concluding that, with the exception of Manicattide, probably all of the investigators, at least in more recent years, have been dealing, either in pure or in impure cultures, with the influenza-like bacillus first described by Spengler and later by Jochmann.

III. PERSONAL OBSERVATIONS.

In undertaking a study of the bacteriology of whooping-cough, my attention was naturally directed to the influenza-like bacilli. In the examinations, however, careful observations were made upon other organisms present with any degree of constancy.

Technique and material.—The sputum coughed up during a paroxysm was obtained in a sterile Petri dish and examined as soon as possible both in smear and culture. As a rule the particle of sputum was washed in several successive dishes of sterile water to remove the contaminating mouth organisms. A part of this was then used for smears and a part for culture. Two smears were made, one of which was stained with Loeffler's methylene blue, and the other with Gram's stain. As a rule I found it useful to counterstain the latter with a very dilute solution of carbolfuchsin. In this manner the Gram-staining and non-Gram-staining organisms could be very clearly differentiated, the latter, which are so numerous in pertussis sputum, taking a faint red stain, while the former appear deep blue or black.

The portion of sputum used for culture, after washing, was introduced into plain broth and thoroughly agitated; four tubes of melted agar at a temperature of 43° C., to which six drops of defibrinated blood had been added, were inoculated with varying quantities of the washed sputum and then plated. In a few of the cases sputum could not be obtained, and swabs of the throat were used. After 24 and also after 48 hours the plates were carefully examined and the various organisms present recorded.

Pigeon-blood agar was used in about one-half of the examinations. In this it was found that the influenza-like organisms, as a rule, grew more abundantly, and the colonies were larger. However, it is not essential, as they will develop on any hemoglobin media. The pigeon-blood is obtained by passing the needle of a Luer syringe into the heart of the bird at a point just above the large breast-bone under the left wing. About 5 c.c. may be obtained at one time from the heart without injuring the pigeon. This blood is at once defibrinated with a wire, and then may be used immediately, or kept in the ice-box for future use.

Bacteriology of the cases.—The sputum of 61 cases of pertussis have been studied in smear and culture. In most cases one specimen

was obtained, but in some cases several specimens were studied. In a number of the cases I examined the sputum in hanging drop, having in mind especially the protozoa reported by several to have been found in this way. Nothing was seen which could be taken for protozoa, except occasionally a ciliated epithelial cell. The leucocytes and pus cells vary a great deal in their appearance, and one might mistake some of these for ameba. Stained preparations frequently show ciliated epithelial cells, and also numerous large, flat epithelial cells and polymorphonuclear leucocytes, but nothing that could be interpreted as protozoa was seen.

A number of specimens of whooping-cough sputum were examined with the ultramicroscope with the view of detecting the possible presence of an ultramicroscopic motile organism. This was done by diluting the sputum in broth and then passing this through a porcelain filter, which would retain all the microscopic particles. With the ultramicroscope many small particles could be seen in the filtrate but they were all non-motile, and were probably particles of proteid. Such filtrates remained permanently sterile when tested on various kinds of media.

Smears made of the sputum showed as a rule both Gram- and non-Gram-staining bacteria. The Gram-staining ones, for the most part, were lanceolate diplococci, frequently encapsulated. It is common to find these adhering to the large, flat epithelial cells so often encountered in the sputum. There is little doubt that they belong to the pneumococcus group. Only rarely is a streptococcus chain observed. Occasionally one meets Gram-positive bacilli which are shown by culture to belong usually to the diphtheria group. Cocci of the staphylococcus type are frequently seen, but are few in number. The non-Gram-staining organisms are usually most numerous, and of these a short, small bacillus, practically indistinguishable from the influenza bacillus, is most common. Sometimes it exists almost alone and in enormous numbers. It never forms threads in the sputum, and occasionally it is seen in the leucocytes in large numbers. Another non-Gram-staining organism, not infrequently met with in smear, is a biscuit-shaped diplococcus which appears to be *M. catarrhalis*. In a few of the cases, fusiform bacilli and spirilla were found in large numbers.

Upon blood-agar plates the varieties of bacteria appear so characteristic that after some experience one can differentiate them with a considerable degree of certainty. The pneumococcus, as a rule, has a typical greenish halo about the colony, and the streptococcus has usually a wide clear zone of hemolysis. Sometimes diphtheria colonies appear practically identical with the streptococcus, though as a rule the colony is whiter and more elevated and the zone of hemolysis not so wide. Not infrequently the zone is absent. The influenza-like organisms form small transparent delicate blue colonies without any zone of hemolysis. Under the microscope, they are nearly homogeneous, with a regular margin. The colonies of *M. catarrhalis* may at first be confused with the influenza-like colonies. There is no zone of hemolysis, the colony is larger, more elevated, and usually darker in color. The margin is irregular and the center is coarsely granular. Some non-hemolytic Grampositive diplococci, occasionally met with in the first 24 hours, may be mistaken for influenza-like colonies, but in smears they are readily distinguishable.

In Table 1 is given a summary of the cases of whooping-cough examined, with data indicating the more important organisms found. Under the various headings the approximate number of organisms is represented by + signs.

Of the 61 cases examined there were 33 males and 28 females; four of them were under one year of age and three were adults. The examinations were made during the fall, winter, and spring of 1904-5. At no time during this period was there an epidemic of influenza. In eight of the cases, bacilli belonging to the diphtheria group were found in the sputum. Most of these organisms had granules, and they usually produced hemolysis on the blood plates. In none of these eight cases were they numerous, and none gave a history of a recent attack of diphtheria, nor did any of them develop it later. Several of these organisms were given to Dr. Hamilton for further investigation. In one case, No. 33, the child had a well-developed, typical case of whooping-cough with no clinical evidence of diphtheria. A swab was obtained from the throat, which upon examination showed numerous influenza-like organisms, but no diphtheria bacilli, in smear or upon the blood-agar plates. Five days later the patient appeared with a well-developed membrane showing on examination many typical diphtheria bacilli, together with some influenza-like organisms.

Under the streptococcus group are included the Gram-positive cocci, whose small, delicate blue colonies have a wide, clear zone of hemolysis about them. In like manner, under the pneumococcus group are included the small colonies appearing in the blood plates, and surrounded by a green zone. While such a differentiation of

TABLE 1.
ORGANISMS FOUND IN PERTUSSIS SPUTUM.

No.	Sex	Age	Duration of Disease When Examined	Influenza-like Bacilli	Micrococcus catarrhalis	Pneumococcus Group	Streptococcus Group	Diphtheria Group	Remarks
1.....	M	1 yr.	1 wk.	+	o	++	++	+	Spasmodic cough. Distinct whoops few days later
2.....	F	3 yrs.	5 wks.	+++	o	++	+	o	Two examinations made Whooping-cough followed diphtheria
3.....	F	1 yr.	5 "	+	o	++	++	o	
4.....	F	3 yrs.	13 "	++	o	++	+	o	
5.....	F	5 "	1 wk.	+++	o	++	+	o	Fusiform bacilli and spirilla present in large numbers. Four examinations made
6.....	M	3 "	1 "	+++	o	++	+	o	
7.....	F	4 "	6 wks.	o	++	+	++	o	
8.....	M	3 "	3 "	+++	+++	++	++	+	Fusiform bacilli and spirilla numerous in smears
9.....	M	8 "	3 "	+	o	+++	+	+	Fusiform bacilli and spirilla numerous
10.....	F	6 mo.	4 "	++	o	++	+	+	Five examinations made before finding influenza-like bacillus. It appeared during the fourth week
11.....	F	9 yrs.	4 "	+++	o	++	+	o	Three examinations made. Influenza-like organisms appeared on 15th day after first whoop noted
12.....	M	2 yrs.	5 wks.	++	o	++	+	o	
13.....	F	9 "	15 days	++	o	+++	+	+	
14.....	F	Adult	1 wk.	++	o	++	+	o	B. mucosus capsulatus present in large numbers.
15.....	F	5 yrs.	6 wks.	+++	o	++	++	o	Six examinations made. Influenza-like organisms appeared during sixth week of disease. The colonies were slightly hemolytic
16.....	F	1 yr.	3 "	+++	o	++	++	o	Sputum not obtainable. Cultures made from throat swab
17.....	F	5 yrs.	1 wk.	+++	o	++	+	o	Two weeks before child whooped examination of throat swab was negative for influenza-like organisms. At this time she had a dry cough
18.....	F	3 "	..	++	o	+++	+	o	Coughed and vomited but did not whoop until five weeks after this examination. Blood culture negative
19.....	M	3 "	5 wks.	++	+	++	+	o	Marked tendency of influenza-like organisms to form long threads
20.....	F	6 "	5 "	++	o	+++	+	+	
21.....	F	6 "	4 "	++	o	++	+	o	
22.....	F	4 "	3 "	+	..	+	++	o	Had whooping-cough in the spring; ceased coughing during summer but began again in the fall. The examination was made at this time
23.....	F	3 "	4 "	++	..	++	++	o	
24.....	F	3 "	3 "	+	..	++	+	o	
25.....	F	2 "	2 "	+	..	++	+	o	
26.....	M	5 "	10 days	+	o	+++	o	o	
27.....	M	7 "	6 mos.	+++	o	++	+	?	
28.....	F	3 "	"	++	o	+++	o	o	
29.....	M	6 "	"	++	..	+++	+	o	Throat swab was obtained for examination several days before whooping occurred
30.....	F	3 "	Several days before whooping occurred	+	..	++	o	o	

TABLE 1—Continued.

No.	Sex	Age	Duration of Disease When Examined	Influenza-like Bacilli	Micrococcus catarrhalis	Pneumococcus Group	Streptococcus Group	Diphtheria Group	Remarks
31.....	M	Adult	1 wk.	++	..	+	++	o	
32.....	F	3 yrs.	2 wks.	o	..	++	++	o	Only one examination possible
33.....	M	6 "	4 "	++	o	++	+	..	A second examination five days after the first showed a typical diphtheritic membrane with numerous diphtheria bacilli
34.....	M	5 "	4 "	o	o	+++	+	o	Only one examination possible
35.....	M	4 "	3 "	+++	..	++	+	o	
36.....	M	4 "	4 "	+++	o	+++	+	o	
37.....	M	4 "	3 "	+++	..	++	+	o	
38.....	F	4 "	4 "	++	o	++	+	o	Obtained pure culture of influenza-like organism by having child cough against plate 10 inches away
39.....	M	5 "	2 "	++++	—	++	+	o	
40.....	M	4 "	3 "	++++	o	++	+	+	
41.....	M	6 "	..	++++	o	++	+	o	Coughed severely, but first heard to whoop two days after examination
42.....	M	7 yrs.	3 wks.	++++	o	+	+	o	Almost pure culture of influenza-like organisms
43.....	F	10 "	Several weeks	++	o	++	+	o	
44.....	M	8 "	"	++	o	++	+	o	
45.....	M	9 "	4 wks.	++	o	++	+	o	
46.....	M	8 mos.	5 "	++++	o	+	o	o	Nearly pure growth of influenza-like organism
47.....	M	7 "	5 "	++++	o	+++	+	o	
48.....	M	5 yrs.	5 "	+	o	+++	+	o	Influenza-like bacilli show marked tendency to thread formation *and phleomorphism
49.....	M	3 "	4 "	o	+++	+	+	o	Only one specimen obtainable
50.....	M	6 "	6 "	+	o	+++	+	o	Nearly recovered when examination was made
51.....	M	7 "	5 "	++	?	++	+	o	
52.....	F	4 "	4 "	+	+	++	++	o	Complicated with syphilis
53.....	M	17 "	4 "	+++	o	+++	++	o	Whooping-cough followed pneumonia
54.....	M	5 "	6 "	+	+	+++	+	o	Very severe vomiting
55.....	F	6 "	6 "	+	++	+++	+	o	Whooping-cough followed pneumonia and empyema
56.....	M	4 "	3 "	+++	o	++++	o	o	
57.....	M	10 "	5 "	++++	o	+	o	o	Examination by throat-swabs
58.....	F	8 mos.	3 "	++	o	++	+	o	
59.....	F	3 yrs.	2 "	+++	o	++	++	o	
60.....	M	2 "	3 days	+	o	+++	++	o	Blood culture was negative
61.....	M	2 "	3 "	+	++	+++	+	+	

pneumococcus and streptococcus may not be absolutely correct, for practical purposes it probably gives an approximately accurate indication of the occurrence of these organisms. As will be seen from the table, both of them are found in practically every case. Streptococci are, as a rule, not nearly so numerous as pneumococci.*

*In the pneumococcus group is included Schottmüller's *Streptococcus mitior seu viridans* inasmuch as it produces green colonies on blood-agar plates. Inulin tests of the various colonies were not made. Since these green colonies were found constantly in the throats of all cases, not only of

Organisms corresponding to *M. catarrhalis*, as described by Pfeiffer, occurred in large numbers in a few cases. They occur in pairs, being indistinguishable in smear from the meningococcus. Usually, when present, they are seen in large numbers inside the leucocytes in the sputum.

Influenza-like organisms.—In these cases attention was particularly directed to the occurrence of the influenza-like bacilli. Usually they were found in the first examination. In this series 44 of the 61 cases required but one examination to find such bacilli. In 12 of the 56 cases in which they were found, two or more examinations were necessary to find them. In five of the 61 cases they were not found, but in three of these only one examination was possible; in the remaining two, two examinations were made in one, and four in the other, with negative results. In two cases the organisms were found several days before the patients had been heard to whoop, but they had at this time a severe cough and expectorated considerable mucus. In six cases where the children were known to have been exposed to the disease, and were showing some symptoms of a dry cough, swabs of the throat were examined for these bacilli, but in none were they found. They all developed typical whooping-cough in from one to two weeks later. As to the length of time these organisms persist in the throat, present data will not suffice. One case that had developed pertussis in the spring and had ceased coughing during the summer, began to whoop again in the fall, i. e., about six months from the first attack. The sputum at this time contained an abundance of the bacilli. A brother of this child, who also had pertussis in the spring and recovered during the summer, but who did not cough in the fall, also showed large numbers of the same bacilli in his throat at this time. The organisms were as abundant in the adults as in the young children. It may therefore be said that these bacilli occur practically constantly in the throats during the spasmodic period, and even slightly before this, but probably not during the initial stage of the disease.

The bacilli were often extremely numerous, in a few cases being nearly in pure culture; this, however, depends largely on how whooping-cough, but of the diseases given in Table 2, as well as in the 20 normal throats examined, they should be considered the normal inhabitants of the throat. It is of interest that organisms of this type are practically absent on the normal nasal mucosa as shown by the examination of the same 20 normal cases that yielded them in abundance from the pharyngeal mucosa.

thoroughly the sputum is washed. In smears of the sputum they sometimes appear as the only organism present. In a few cases the leucocytes in the sputum were crowded with them, but generally they were outside the cells.

Morphology.—The organism is a small, short, non-motile bacillus, which does not take Gram's stain, does not have a capsule, and does not form spores. It stains more deeply at the ends, especially with methylene blue, and therefore may be mistaken for a small diplococcus. It stains especially well with dilute carbolfuchsin. In sputum the bacillus is nearly always single, but occasionally two or three bacilli may be seen in a chain. Their morphological characteristics in sputum are very uniform. In the culture, however, it is quite different. In many of the strains there is a marked tendency to chain and thread formation. Frequently the organisms occur as coccus forms and in thick, curved, or S-shaped threads. Sometimes threads may be seen reaching across the field of the microscope, and much thicker than the single bacillus. As was stated, they are never seen in smears direct from the throat, but may occur in the first generation in the blood plates, or develop later. They are usually more marked in a culture a few days old. When one meets these for the first time he is convinced that he has a mixed culture or some entirely different organism. These peculiar irregular forms agree well with Pfeiffer's pseudo-influenza organisms, which he isolated from cases of broncho-pneumonia. However, this fact should be emphasized, that many of the strains do not show this tendency to any extent under any circumstances, and, so far as this point is concerned, agree with Pfeiffer's description of the real influenza bacillus. As regards the size of the bacillus, the various strains all appear about the same in sputum, and, compared with the few influenza bacilli that I have been able to obtain, show practically no differences. In cultures, however, bacilli of various strains from whooping-cough vary in size so much among themselves that a comparison is difficult. Certainly many of the whooping-cough strains are as small as organisms from typical clinical cases of influenza.

Cultural characteristics.—The organisms from pertussis correspond in their cultural characteristics in every way to Pfeiffer's bacillus. It is a strict aerobe. On plates the colonies are small, moist and

dew-drop-like. They have a delicate blue color and do not hemolyze. In one case (No. 15) only, did I observe a very narrow but distinct clear zone of hemolysis about the colonies. This persisted through two generations, and then disappeared and could not be obtained again. The colonies, if not very numerous, especially on pigeon blood, will sometimes grow very large. Some have been observed over 2 mm. in diameter. As a rule they are $\frac{1}{2}$ mm. or less in diameter, and not infrequently, if numerous on the plate, require the aid of a hand lens to be seen. They do not increase in size after from 24 to 36 hours. Upon blood-agar slants they produce a delicate growth and the colonies remain discrete.

In every case, after getting the organism in pure culture on blood media, it was tested on several kinds of non-hemoglobin media, usually Loeffler's blood serum, plain agar, and milk. Many of the strains were tested on serum agar, serum broth, ascites agar, and various kinds of sugar media. In none of these did any of the organisms develop. Particular attention should be called to the fact that there is not infrequently in human serous fluids a small amount of hemoglobin, and if such fluids are used growth will occur. This fact may explain some of the reports in the literature, such as that of Elmassian, of organisms from pertussis cases growing upon serum media. To be sure of proper results, one should examine such fluids with a spectroscope before using.

Media to which yolk of egg was added did not yield any growth. Bile was added to agar, and some growth was obtained with this in the first transplantation, but continuous growth did not occur. The organism in pure culture did not grow on hematin agar. Upon CO-hemoglobin they grow as well as upon ordinary oxyhemoglobin. Inasmuch as oxygen is necessary for their growth, the CO-hemoglobin, made by passing a stream of CO through blood for half an hour, was necessarily exposed to the air, and a small amount was consequently changed back to oxyhemoglobin, which might explain this growth. The abundant growth suggests, however, that the CO-hemoglobin may also be available, though it is perhaps impossible to prove this. Growth is obtained in liquid media to which a drop or two of blood is added. Plain broth plus a small amount of pigeon's blood is a good medium. The blood corpuscles soon sink to the bottom, leaving

a perfectly clear fluid above. When inoculated, this becomes turbid as a result of the growth, such a culture being very convenient for inoculations. The bacilli grow in milk, and also in the various kinds of sugar media, if a little hemoglobin is added, but no change occurs in any of them.

Only a very small amount of hemoglobin is required for the growth of these bacilli. One may dilute blood with sterile water many times, and if a few drops of this laked solution is added to agar a clear transparent medium, scarcely colored at all, is obtained, upon which the bacilli grow in abundance. This is a very convenient medium to use for their culture. The organism is rather delicate and must be transferred every five days to make sure of growth. They seem to live somewhat longer in liquid than on solid media. They are very susceptible to drying, and a temperature of 42° C. for a few hours is fatal. The bacilli have been observed through many successive generations without undergoing any essential change. Some strains have been transplanted every four or five days for six months, and their morphology and cultural characteristics remained unaltered.

These bacilli are thrown out into the air in large numbers during the coughing spell of the child, and if one is in front of the patient he is sure to inhale some of them. In five cases blood-agar plates were held in front of the patient from 6 to 12 inches away during the spasm, and in every instance the bacilli were isolated. There are always other organisms on the plate, and usually the colonies are not pure, for the bacilli are carried out from the throat in particles of spray, which one would expect to contain more than one kind of bacteria. With babies, where it is difficult or impossible to obtain sputum, examination for the bacilli may be made in this way.

Symbiosis.—Grassberger¹ has called particular attention to the occurrence of very large influenza colonies in the immediate neighborhood of colonies of other bacteria, when grown with them on blood-agar plates. He worked especially with staphylococcus, but the same effect is obtained when influenza bacilli are grown with many other varieties. He observed influenza colonies as large as 4 mm. in diameter, grown in this manner, which in pure culture are usually only $\frac{1}{2}$ mm. or less. Jochmann also observed this same

¹ *Ztschr. f. Hyg.*, 1897, 25, p. 453.

phenomenon in connection with his *B. pertussis* Eppendorf. It is well known that influenza bacilli will live and develop through several generations on non-hemoglobin media, when grown in mixed culture. Pfeiffer, before he discovered the use of hemoglobin for influenza culture, noted the growth of influenza bacilli on agar smeared with sputum, but could not successfully transplant them on this medium in pure culture. M. Neisser¹ obtained mixed cultures of *B. xerosis* and *B. influenzae* from conjunctivitis in measles, and grew them through 20 generations on plain agar. Strains of hemophilous bacilli from throats of whooping-cough, measles, and scarlet fever were in like manner carried through many generations in mixed culture. With dead *B. xerosis* or its extracts, no growth occurred. He says the presence of a living organism is necessary to produce this symbiotic effect.

Various attempts have been made to explain the mechanism of symbiosis. Ghon and Preyss² think it may be due to a reduction of the hemoglobin or hematin by some substance produced by the foreign organisms, thereby rendering the iron more available. Luerssen³ found the favorable substance residing in the cell body of the bacteria, and not in any product. He also claimed that filtrates, made after several days' growth, were favorable for the development of the influenza bacillus.

Frequently, in the blood-agar plates inoculated from the whooping-cough sputum, there was observed a conspicuous cluster of influenza-like colonies surrounding other colonies present on the plates, such as those of streptococcus, staphylococcus, and pneumococcus. They were, in this location, not only larger, but apparently more numerous, than when farther away from the foreign colonies. In order to obtain such an arrangement, it seems necessary to have a large number of influenza colonies in the plate, and a few of the foreign organisms. When the former colonies are numerous, they are often so small that a hand lens is necessary to see them; but around the foreign colonies, as a result of some favorable influence, they become much larger and are more easily visible. This appearance may be easily obtained by sowing a blood-agar plate densely with the

¹ *Deutsche med. Wchnschr.*, 1903, 29, p. 462.

² *Ibid.*, p. 434.

³ *Centralbl. f. Bakt.*, Abt. I, Orig. 1904, 35, p. 531.

pertussis bacillus, and sparsely with some other organism, such as staphylococcus. In 24 hours the symbiotic relationship is usually evident.

If pertussis sputum containing large numbers of the influenza-like bacilli, as it commonly does, is spread on plain agar or Loeffler's blood-serum, these bacilli will often grow in abundance. Other germs are always present under these circumstances, and undoubtedly their growth has much to do in causing the influenza-like bacilli to develop on the hemoglobin-free media; for, in sterile sputum alone, the latter do not grow. If transferred thus in impure culture upon plain media, they often survive several generations, and their growth may be easily determined by making blood-agar plates at the time of each transplantation. It was practically this technique that Czaplewski and Hensel, and others who obtained similar results later, used to isolate their bacillus, and one is strongly tempted to believe that they have thus been cultivating in impure culture the same hemophilous bacillus found by Spengler and Jochmann. As already stated, this was Spengler's explanation of their results. Such an interpretation seems far more reasonable than the possibility that Czaplewski's organism, found as frequently as he reported, was an entirely different organism from the hemophilous bacillus found by so many.

The growth of the several strains of influenza-like bacilli from pertussis was tested upon various media, to which were added dead bacteria (staphylococci, streptococci) and filtrates of cultures; the results were uniformly negative. All of the experiments performed indicated that the symbiosis is dependent on the presence of the living organisms.

The symbiotic relation of other bacteria to the influenza-like organisms is shown by the increased pathogenicity of the latter for animals. This will be more fully discussed under the head of animal experiments.

IV. ANIMAL EXPERIMENTS.

Literature.—The results obtained by the inoculation of whooping-cough virus into animals by various investigators have for the most part been negative. The older reports of producing the disease in dogs can hardly be considered seriously. Afanassiew reported that

he produced a real whooping-cough in animals by the inoculation of his organism, but these results have never been substantiated. Ritter's animal experiments were not conclusive. Injected into the circulation, his diplococcus produced no effect. Czaplewski and Hensel, and also Zusch, obtained no results in animals with their organism. Koplik reported that intravenous inoculation with his bacillus produced suppurative arthritis, that 5 c.c. of culture inoculated into white mice killed in one week, and that the organisms were present in the blood; but that inoculation of guinea-pigs and rabbits with sputum gave negative results. Vincenzi's results were negative. Elmassian, with his organism, produced death in young guinea-pigs by intraperitoneal inoculation in 24 hours or less, and isolated the bacillus from the fluids and heart's blood; there was marked phagocytosis. Intravenous injection into rabbits produced some cachexia, while pigeons and mice did not react. Manicatide obtained no results by inoculating his bacillus "z" into the nose and trachea of guinea-pigs, cats, dogs, and monkeys. Jochmann and Krause were not able to produce any effect either with sputum or pure cultures of their bacillus, when inoculated into the nasal cavities, throat, or peritoneum of animals. On the other hand, Smit found Jochmann's bacillus pathogenic for young guinea-pigs by peritoneal injections, and that it also produced lameness, general infection, and death in rabbits by intravenous injection. He also produced double-sided conjunctivitis in a rabbit, and obtained the bacillus in the exudate. A distinct reaction occurred in the horse when injections were made with the bacillus for immunizing purposes. Wollstein reports negative results with the influenza-like organism from intraperitoneal and subcutaneous inoculations in white mice, guinea-pigs, and rabbits, and also from a subdural inoculation in a rabbit.

For comparison with the above results, we may briefly refer to results obtained with Pfeiffer's bacillus. Inoculation experiments with the sputum of influenza patients and with pure cultures of the bacillus upon guinea-pigs, rats, mice, and doves are generally without result. In rabbits and monkeys some symptoms have been produced. Pfeiffer, by injecting the bacilli into the lungs of monkeys, observed symptoms simulating those of influenza, and by intratracheal injection of the bacilli killed a monkey in eight hours. There being no

anatomical change visible, this latter result was attributed to the toxic products—surely a most doubtful conclusion. In rabbits, by intravenous inoculation of living bacilli, dyspnea and marked weakness were produced, which Pfeiffer also attributes to toxins, because the same result was produced with dead bacilli. Kolle and Delius substantiated these results, and also showed that influenza bacilli would develop in the peritoneal cavity of animals, especially guinea-pigs. The exudate contained many bacilli, partly free and partly in the cells. They were able to raise the virulence of the bacilli by successive animal inoculations, and in filtrates of cultures obtained a specific but very labile poison. Jacobson¹ increased the virulence of the influenza organism by simultaneous injection with streptococci, and Slatineano² raised the virulence by injecting lactic acid at first and one-half hour later the influenza bacilli.

Experiments on lower animals.—The influenza-like bacilli isolated from the whooping-cough patients in this series showed on the whole a low degree of virulence for animals; however, some very definite results were obtained. Guinea-pigs were found most satisfactory for this purpose. White rats are susceptible, but less so than guinea-pigs; rabbits, especially young ones, are also susceptible when inoculated intraperitoneally. But few inoculations were made in these animals, however. Two blood-agar cultures inoculated intraperitoneally into guinea-pigs will rarely kill in 24 hours. Usually the animal is sick the next day after inoculation, but will gradually recover. If the amount is increased, death will usually occur in 24 hours or less, and from the peritoneal cavity, pleural cavity, and heart's blood, the bacilli are obtained in abundance. In the blood the bacilli are so numerous at times that they may be found without great difficulty in smear. A few drops, allowed to run over the surface of an agar slant, usually shows an abundance of colonies in 24 hours. Blood removed from the heart before death also shows the presence of bacilli, so that their occurrence is not due to a postmortem invasion, but there seems to be an actual multiplication of them in the living blood.

When the animal dies in 24 hours or thereabouts after inoculation into the peritoneal cavity, the peritoneal and pleural exudates are clear, serous in character, and soon clot on standing. The bacilli

¹ *Archiv de méd. expér.*, 1901, 13, p. 425.

² *Comp. rend. de la Soc. de Biol.*, 1901, 51, p. 850

cal irritation. Czaplewski states that, while working with his organism, he was taken with a coryza accompanied with some cough, which lasted for about a week. He obtained his bacillus from his nose and throat in large numbers.

Human inoculation.—As no attempts are on record to inoculate man directly with the influenza-like bacillus from pertussis, it seemed desirable to make such a test with the view of determining its possible pathogenicity. A young man, who, according to his statement and also that of his relatives, had never had whooping-cough, agreed to submit to inoculation with hemophilous organisms isolated from the pertussis cases. This was done with his full consent, and he was informed concerning the character of the material inoculated and the possible results anticipated. He was healthy in every way, and an examination of his throat made previous to the inoculation showed only the usual bacterial flora of the normal throat. There were no influenza-like organisms present. Pure blood-agar cultures, 24 hours old, of influenza-like bacilli, isolated from an uncomplicated case of whooping-cough, were smeared upon the nasal mucosa and the tonsils. On the second day, almost exactly 48 hours after the inoculation, the patient complained of a chilly sensation, a cold sweat, some headache and weakness. His temperature rose from normal (98.4° F. the day previous) to 100.2° F. The same evening it registered 100.1° F. The next morning it was normal again, but rose during the day to 99.5° . The third day his temperature was practically normal and continued so from that time on. He complained somewhat of headache and of feeling unwell, and on the second morning said he coughed a little. His throat, upon examination when the first symptoms appeared, was slightly hyperemic; the next morning there was present a thick layer of mucus upon the pharyngeal wall, and from this time on he coughed, or rather hacked up large quantities of a stringy, tenacious mucus. This condition continued, becoming gradually less marked, and was still apparent at the end of four weeks. After the first few days he felt perfectly well, complaining only of slight discomfort in the throat and the light cough. This cough was not spasmodic and did not resemble whooping-cough. The leucocyte count on the second day was 9,200.

Bacteriological examination of the mucus from the throat, obtained

on the first day of symptoms revealed almost a pure culture of the influenza-like bacilli. They were present both in the throat and on the nasal mucosa, in enormous numbers. Direct smears of the expectorated mucus showed practically no other organism. They were identical in every detail with the organisms inoculated. Examinations of the throat were made every few days for four weeks, after which time the patient was not accessible. The number of organisms present became gradually less, but at the end of that time they were still present in considerable numbers.

From this experiment we must conclude that this organism develops in the human throat when implanted there, and is capable of causing a distinct reaction. The fact that pertussis was not produced by this one inoculation by no means excludes this organism as an etiologic factor in the disease. For, in a case of this kind, it is impossible to be absolutely sure that the individual has never had the disease, and adults at this age may be immune. The result does indicate surely that this organism, present almost constantly as it is in pertussis, and capable of producing such reaction in an adult, must be of some significance in the disease, and is at least not always a harmless saprophyte.

V. INFLUENZA-LIKE ORGANISMS IN OTHER DISEASES AND IN NORMAL THROATS.

The occurrence in other diseases of what appears to be the same organism, morphologically and culturally, is of interest. A number of examinations were made of sputum and throat swab by the same method as that used in whooping-cough, from cases of measles, acute influenza, epidemic cerebrospinal meningitis, bronchitis, varicella, and from normal throats.

Measles.—Twenty-two cases of uncomplicated measles were examined. The sputum was obtained in most of the cases, but in a few this was impossible, and throat swabs were used. All were examined during the stage of eruption, and most of them had at the time the usual cough present early in the disease. Only one examination was made in each case. The influenza-like bacilli were isolated in pure culture in 13 of the 22 cases. In four of the cases they were the predominating organism; in some cases they were very few

in number. Streptococci and pneumococci were found in every plate, the latter always predominating. The *M. catarrhalis* was found in 10 of the cases, being extremely abundant in two. Diphtheria bacilli were obtained from the throats in two cases. They were large curved bacilli containing large granules, and the colonies showed a distinct, clear zone of hemolysis. One of the cases had a profuse nasal discharge during convalescence. In this material were found by culture pneumococci, streptococci, a few diphtheria bacilli and a considerable number of influenza-like bacilli. Four of the cases were complicated with otitis media, and the pus was examined in each case. In one case the influenza-like bacilli were obtained nearly pure, there being present also a few streptococci. In this case the bacilli had been obtained from the throat about a week previously; 11 days later the pus was again examined, and showed no influenza bacilli, but nearly a pure culture of a bacillus of the diphtheria group. From the other three cases streptococci were obtained, pure in one of them, and streptococci and diphtheria bacilli, mixed, in the remaining two.

Clinical influenza or grippe.—The sputum was examined from 17 cases that were diagnosed clinically as acute influenza. They occurred at a time when there was reported to be an epidemic of grippe in the city. In only three cases were the influenza bacilli found, and in only one of these were they very numerous. Streptococci were very conspicuous in these cases, and while not so numerous as pneumococci, they were more abundant than in the other groups examined.

Epidemic cerebrospinal meningitis.—In five cases of typical cerebrospinal meningitis the influenza-like bacilli were found either in the nose or throat of four. The case in which it was not found had been sick for three weeks when examined, and at that time showed no nasal or throat symptoms. The other four cases were examined early in the disease, and showed some nasal and throat symptoms. In one of these the meningococcus was isolated from the nasal cavity and also the sputum, and in this same case the influenza-like bacilli were also present in large numbers, being by far the predominating organism. The meningococcus was cultivated from the cerebrospinal fluid in each of the five cases.

Bronchitis.—This comprises a more or less miscellaneous group of cases. All had bronchitis, some for a long period, while in others it occurred in a more acute condition. In some cases it was a complication of some other disease. The influenza bacilli were found in five of the 12 cases examined. In two of these cases they were very numerous, by far exceeding all other organisms present. One case in this series was a typhoid which began with a marked bronchitis. Here was found, in practically pure culture, the *M. catarrhalis*, but no influenza-like organisms. This micrococcus was found in only one other case of this group.

Varicella.—Throat swabs from 11 cases of varicella in young children were examined in the usual way. All of the children had a slight cough, and three had at the time of the examination a gonorrheal vaginitis. The influenza-like bacilli were isolated in seven of the eleven cases; in two they were numerous. Streptococci were found in eight, diphtheria bacilli in one, and the *M. catarrhalis* in all of the cases. The latter organism was very numerous in two cases in which the influenza-like organisms were absent.

Normal throats.—For these examinations swabs were made from the posterior wall of the pharynx and were for the most part obtained from medical students. In 20 cases the influenza-like bacilli were found twice; in neither case were they numerous. The throats of these two individuals appeared normal, and they gave no history of any throat trouble for at least three months previously, remaining perfectly well for several weeks at least following the examination.

Table 2 gives a summary of the occurrence of influenza-like bacilli in the diseases investigated. It should be noted that in all the diseases except whooping-cough but one examination was made. In this disease, in a number of cases, several examinations were made before finding the bacilli. It is therefore quite probable that if more examinations were made in the other conditions the percentage of positive results would have been higher.

The bacilli from these various sources were carefully observed and their properties tested. Every organism was transplanted to non-hemoglobin media, and not one was found which would grow. Particular attention was given to the test on ascites-agar and serum-agar, as so many reports have been made that organisms of this kind

TABLE 2.
OCCURRENCE OF INFLUENZA-LIKE BACILLI IN VARIOUS DISEASES AND IN NORMAL THROATS.

	No. of Cases Examined	Influenza- like Bacilli Present	Influenza- like Bacilli Not Found	Percentage of Positive Findings
Whooping-cough.....	61	56	5	92
Epidemic cerebro- spinal meningitis....	5	4	1	80
Varicella.....	11	7	5	63
Measles.....	22	13	9	59
Bronchitis.....	12	5	7	42
Influenza (grippe)....	17	3	14	18
Normal.....	20	2	18	10

occur which will develop upon these media. In no instance did a continuous growth occur when proper precautions were taken to use fluids free from hemoglobin. Their cultural characteristics were identical, and morphologically there were no constant differences between them. In every group strains were found with a tendency to marked thread formation, and there was also considerable variation in the size of the various strains, but nothing was observed that would characterize any particular group. Animal experiments showed that the bacilli from the different groups possessed about the same pathogenic power as those isolated from whooping-cough cases. Even those isolated from normal throats manifested the same low degree of virulence as the other strains. A large quantity of the bacilli is necessary to produce death, and the symptoms are not those of a toxemia but of a general invasion, with numerous bacilli in the blood.

Literature.—In the literature there are numerous references to the occurrence of hemophilous bacilli in many infectious diseases and diseases of the respiratory tract. They have usually been referred to as influenza bacilli (Pfeiffer's bacillus), occasionally as pseudo-influenza organisms, and for the most part have been looked upon as secondary invaders, except in influenza, in which the hemophilous bacilli have pretty generally come to be considered as the specific organism. Pfeiffer, in 1893, in his classical work on influenza, was the first to announce this, and since then an enormous mass of literature has accumulated, most of which tends to substantiate the idea that his bacillus is the specific cause of this disease. If the evidence is carefully examined, however, it is found

that in reality the specificity of this organism does not rest upon any too sure foundation. It does not meet Koch's requisites. In many epidemics the bacilli are constant in the throats, being often present in large numbers; and they have been obtained from abscesses and numerous other lesions in the body. In postmortems the organisms are found in large numbers, especially in the respiratory passages and the lungs. It is somewhat questionable whether the influenza bacillus has ever been obtained from the blood during life. Canon's results are absolutely discredited by Pfeiffer himself, and the work of Jehle, Isambert, Rosenthal, and others needs further confirmation. The pathogenicity of the bacilli for animals is very low, and the experiments of Pfeiffer in regard to the presence of toxins are not at all convincing—though the more recent work of Kolle and Delius and Slatineano indicate their occurrence. These facts are hardly sufficient to demonstrate beyond question that the organism is specific, and that it is not merely a secondary invader.

It is interesting that the French especially have always been skeptical about the etiologic rôle of Pfeiffer's bacillus in influenza; and many data have been presented in opposition to the view that it is specific for this disease. Elmassian,¹ in 1899, could not distinguish between organisms obtained from whooping-cough, acute bronchitis, pulmonary tuberculosis, and pneumonia. He considered them all identical with Pfeiffer's bacillus of influenza, and said that it was not proved that this bacillus was the specific cause of grippe, but should be considered only the probable cause. Rosenthal² quotes Metchnikoff as saying that Pfeiffer could not find the influenza organisms in an epidemic of grippe in 1899. He thinks it an ordinary microbe of the pathologic flora of the lungs, and not specific for grippe. Sacquépée³ studied a typical and very severe epidemic of grippe in a garrison, and found Pfeiffer's bacillus in only the latter part of the epidemic (February). At other times he found a Gram-negative, motile, typhoid-like bacillus, and also pneumococci and streptococci. He thinks that the influenza bacillus is not specific, and that grippe may not be due to any specific microbe. Bezançon and Israëls de Jong,⁴ in an epidemic of grippe, studied the expectorations of 25 cases bacteriologically, and found Pfeiffer's bacillus to be very rare. They call special attention to "Micrococcus catarrhalis" and to "paratetragene zoogléique" which were very common. They found pneumococci, streptococci, pseudo-diphtheria, pneumobacilli, and rarely staphylococci. They conclude that grippe is not caused by Pfeiffer's bacillus, but is due to a variety of organisms of exalted virulence. Kleinenberger,⁵ in 27 cases of epidemic influenza, found the influenza bacillus only eight times. The *M. catarrhalis* was present in nearly every case. Lord,⁶ of Boston, has

¹ *Ann. de l'Inst. Past.*, 1899, 13, p. 621.

² *Compt. rend. de la Soc. de Biol.*, 1900, 52, p. 266.

³ *Arch. de méd. expér.*, 1901, 13, p. 562.

⁴ Reviewed, *Bull. de l'Inst. Past.*, 1905, 3, p. 372.

⁵ *Deutsche med. Wchnschr.*, 1905, 31, p. 575.

⁶ *Boston Med. and Surg. Jour.*, 1905, 152, pp. 537 and 574.

studied non-epidemic infections of the respiratory tract, and has clearly shown the similarity, both clinically and pathologically, of the infections due to influenza bacilli and other organisms, such as pneumococcus and *M. catarrhalis*. Von Jaksch¹ has described a condition, under the name of pseudo-influenza, which is indistinguishable clinically from influenza, but which is not caused by the influenza bacillus. He concluded that it was due often to a streptococcus invasion, because of the large number of these organisms found; in some the cause could not be determined, but he was sure it was not due to influenza infection.²

The results of these investigations correspond very well with the findings in the series of influenza cases reported in this paper. While it is undoubtedly true that in many epidemics of influenza Pfeiffer's bacillus exists constantly in the nasal and oral excretions in very large numbers, and to the exclusion largely of other bacteria, and that its presence corresponds with the course of the disease, it is equally true that there occur epidemics, and also sporadic cases, of a condition which appears to be identical with influenza clinically, but which shows Pfeiffer's bacillus only occasionally, and other organisms, such as pneumococci, staphylococci, and *M. catarrhalis*, very commonly and in large numbers. The most rational interpretation of these facts seems to be that there are a number of organisms which may give clinically the same picture, and which may exist in the respiratory passages either in nearly pure culture; or they may occur together.

The fact that many cases, indistinguishable clinically from acute influenza, occur both in sporadic and epidemic form not associated with the influenza bacillus, should be more widely known among the medical profession at large. For ever since the great epidemic of 1889-90, almost everything bearing any resemblance at all to influenza is assumed, without a bacteriologic examination, to be an influenza infection. Attention should be called also to the fact that smears of oral and nasal excretions are very unreliable in the diagnosis of this disease, and that, to be sure of an influenza infection, cultures on blood-agar plates should be made. Clinical data, there-

¹ *Berl. klin. Wchnschr.*, 1899, 36, p. 425.

² Since this paper was written an article upon influenza infections by Jochmann (*Deutsches Arch. f. klin. Med.*, 1905, 84, p. 470) has appeared. In 36 cases of epidemic influenzas he was able to find influenza bacilli in only 13. Pneumococci and streptococci were very common. He thinks that the clinical picture of acute influenza may be produced by organisms other than the influenza bacilli; especially the pneumococcus, streptococcus, and perhaps the *M. catarrhalis*. He discusses the occurrences of influenza-like bacilli in the infectious diseases and says the organisms from whooping-cough are indistinguishable morphologically and biologically from the bacillus of Pfeiffer. His results agree very well with those given in this paper.

fore, unless accompanied by careful bacteriological examinations, are of little value. This is mentioned because in the literature, even of late years, so much is written upon influenza without definite knowledge of the presence of influenza bacilli.

Besides influenza, in which hemophilous bacilli occur, and are the probable, but not the demonstrated, cause of the disease, there are numerous other diseases in which hemophilous bacilli, usually called influenza or pseudo-influenza organisms, have been frequently found. A brief résumé of the more important literature upon this point will here be given.

Pfeiffer, as already stated, found what he called pseudo-influenza bacilli in broncho-pneumonia, and distinguished them from influenza by their greater size and their tendency to form threads. Numerous writers since that time have noted the marked tendency to form threads in bacilli from cases of influenza, and have also noted considerable variation in size in various strains of such organisms. Also, attention has been called to the occurrence in other conditions of bacilli not differing morphologically from Pfeiffer's influenza bacillus. Pfeiffer's distinction, therefore, between the bacilli from influenza cases and those from other sources does not appear to hold true, and the pseudo-influenza, for the present, must be considered identical with the influenza bacillus. Elmassian found influenza bacilli in acute bronchitis, tuberculosis, and pneumonia. Rosenthal¹ found them in 15 out of 19 cases of broncho-pneumonia, nearly pure in two. Susswein² obtained them in 10 out of 21 cases of measles. Lo-d,³ in a very valuable paper, reports his findings in diseases of the respiratory tract, chiefly acute and chronic bronchitis. He obtained influenza bacilli in every way corresponding to Pfeiffer's bacillus in 56 out of 186 cases (30 per cent), and in 47 cases the bacilli were in overwhelming numbers. These cases were examined at a time when there was prevalent no epidemic of any acute respiratory disease. Smith⁴ found influenza bacilli five times in 73 cases of lobar pneumonia. Leiner⁵ obtained them constantly from the bronchial secretion and lungs of 11 postmortem cases of diphtheria. Neisser⁶ found them in cases of measles, scarlet fever, and whooping-cough. Jehle⁷ found influenza bacilli in postmortems as follows: In 48 cases of scarlatina, 19 times in the lungs, six times in the tonsils, and 22 times in the blood; in nine cases of varicella, nine times in the lungs and five times in the blood; in 24 cases of pertussis, 24 times in the lungs and 12 times in the blood; in 15 cases of diphtheria, nine times in the lungs and five times in the blood. Liebscher,⁸ in 57 cases of measles, found influenza bacilli 11 times, and in 60 cases of scarlatina found them three times. He examined only the nasal secretions. Auerbach⁹ found, in swabs from the tonsils and larynx of 700 cases of acute infectious diseases, the influenza bacillus 12 times in diphtheria, three times in scarlatina, six times in what he calls diphtheria-scarlatina, seven times in diphtheria-morbilli, and 10 times in anginas. Kleinenberger¹⁰ found hemophilous

¹ *Compt. rend. de la Soc. Biol.*, 1900, 52, p. 226.

² *Wiener klin. Wchnschr.*, 1901, 14, p. 1149.

³ *Brit. Med. and Surg. Jour.*, 1905, 152, p. 537.

⁴ *Jour. Boston Soc. of Med. Sc.*, 3, p. 274.

⁵ *Wiener klin. Wchnschr.*, 1901, 14, p. 1001.

⁶ *Deutsche med. Wchnschr.*, 1903, 29, p. 462.

⁷ *Ztschr. f. Heilkunde*, 1901, 22, p. 190.

⁸ *Prager med. Wchnschr.*, 1903, 28, p. 99.

⁹ *Ztschr. f. Hyg.*, 1905, 47, p. 259.

¹⁰ *Deutsche med. Wchnschr.*, 1905, 31, p. 575.

bacilli in 18 out of 25 cases of pertussis, upon the conjunctiva in three out of nine cases of measles, once in the heart's blood in measles postmortem, once in scarlet fever and once in a case of urethritis. So far as I have been able to determine, there are no reports in the literature upon the occurrence of influenza bacilli in normal throats.

To sum up the literature, the results agree very well with the data given in Table 2, so far as they are comparable. Süsswein's results in measles and Lord's results in bronchitis correspond very closely with those in the table. The findings in influenza agree in general with those obtained by many investigators, especially the French. I do not know of any positive findings in the literature of influenza bacilli in the nose or throats of cases of epidemic meningitis. All the results taken together indicate that we have in these bacilli an organism occurring very commonly in a large number of diseases, and undoubtedly present, at times at least, in all inflammatory conditions of the respiratory passages whatever their character may be. The evidence is conclusive that this organism at times exists as a saprophyte, as indicated by its appearance in normal throats, and that it is also capable of causing inflammatory changes, as shown both by human and by animal experiments, and by the vast amount of pathologic data. The all-important question is, whether we are dealing in all these various conditions with the same organism, varying only in its degree of virulence, or whether we have to do here with a group of very closely related organisms. Morphologically, tinctorially, and culturally, from the results obtained from the various strains isolated in the cases here reported, and in the cases reported in the literature, we must conclude that we have no means of distinguishing them. Reasoning from analogy, we should expect a group, as occurs with so many of the other bacteria; and the chief problem now before us is the careful application of every possible means, particularly in the way of biological experiments, to differentiate the organisms. Already considerable evidence from this point of view exists, but it is conflicting. Wollstein's experiments upon agglutination with the organisms from influenza and pertussis are extremely important and suggestive. They are in accord with results obtained by Cantani¹ with the influenza bacillus, but are opposed, directly or indirectly, to the results obtained by several investigators (Neisser, Smit, Lord, Jehle, Meunier).

¹ *Ztschr. f. Hyg.*, 1903, 42, p. 505.

It is perhaps worth while at this time to call attention to the fact already noted by Jochmann, that whatever may be said in favor of the influenza bacillus as the cause of influenza, practically the same thing may be said in favor of the organism isolated from the cases of pertussis as the cause of that disease. The constant occurrence of the organism at the apparent seat of disease in practically all uncomplicated cases, the parallelism between the general course of the disease and the relative abundance of the organisms, the presence of the organisms in the lungs and various secretions in postmortems, the low pathogenicity of the organism for animals—all these hold true for one equally as well as for the other. The medical world, with few exceptions, has come to recognize the influenza organism as specific for this disease, but it surely is not yet ready to accept the cause of whooping-cough as settled. This fact may be interpreted in two ways; it may be used to indicate upon how insecure a foundation the specificity of the influenza bacillus rests, or it may be used as an argument in favor of the specificity of the pertussis organism. Evidently, with the present data at hand, it is impossible to settle either question absolutely.

Pertussoid, pseudo-pertussis, etc.—A few words may be said concerning a condition variously referred to in the literature as a whooping-cough-like cough in influenza, pertussoid, pseudo-pertussis, etc., by Leichtenstern, Forchheimer, Filatow, Guidi, Pestalozza and others. From the description given, it would appear that these conditions are intermediary forms between influenza on the one hand, and whooping-cough on the other. This is certainly suggestive in view of the fact that bacilli so nearly alike, if not identical, are found so constantly in both these diseases. Forchheimer¹ was able to find the influenza bacillus in but few of the cases, but found streptococci constantly.

I have had an opportunity of examining only two cases which might come under this class. Both were adults, and both had come in contact more or less with whooping-cough patients. Each had a very severe cough, lasting several weeks, spasmodic in character but without distinct whoops. In the sputum of both, influenza-like bacilli were found, quite numerous in one, but very few in the other.

¹ *Archives of Pediatrics*, 1900, 17, p. 801.

In the former were also many streptococci, while in the latter there was nearly a pure culture of pneumococcus. A careful study, with particular reference to the influenza-like bacilli, should be made of the flora of the oral and nasal secretions of a large series of such cases, and especially those cases of cough occurring in parents or persons associated with children having pertussis, and often referred to as a sympathetic cough, and the like.

VI. NOMENCLATURE OF THE BACILLI.

Before concluding, it may be well to discuss briefly the nomenclature of the bacilli of this class. As a general term for organisms of this type, which only grow upon hemoglobin media, "*Bacillus hemophilicus*," used so generally by the French, certainly seems appropriate, inasmuch as it describes their most important characteristic. Such a term would include Pfeiffer's bacillus, the pseudo-influenza bacillus, all the bacilli described as influenza-like or as influenza bacilli found in whooping-cough, measles, scarlet fever, diphtheria, pharyngitis, varicella, bronchitis, tuberculosis, etc., Müller's trachoma bacillus, and a few other organisms having this property and isolated from various sources. Should differences be detected between the various organisms, as in all probability there will be, at least between some of them, special names may then be assigned them; this has been done, by those who believe that there are detectable differences, with organisms from influenza and whooping-cough. As for the last-mentioned organism, if the names of individuals are used to designate it, it should be known either as Spengler's bacillus or the Spengler-Jochmann bacillus. After reading Spengler's description of this organism, it is difficult to see how anyone can believe that he was dealing with an organism different from that described by Jochmann, as the latter writer contends. Spengler called this organism the pertussis bacillus, and if this organism is proved beyond question to be specific for the disease and different from *B. influenzae*, this term should be used, and Jochmann's term, "*Bacillus pertussis* Eppendorf," discarded. However, until these organisms are definitely distinguished from each other, it would seem that the special term, *Bacillus influenzae*, should be used for all.

VII. SUMMARY AND CONCLUSIONS.

1. In the sputum of pertussis patients there is, almost constantly, an organism which, morphologically and culturally, is identical with the influenza bacillus. It is usually in greater abundance than any other organism of the sputum.

2. This organism is most abundant during the spasmodic stage of the disease. It has been found several days before the whoops began, and as long as six months after the disease.

3. During the coughing spasms it is thrown out in the surrounding air in large numbers.

4. Alone, its pathogenicity for animals is low; when associated with other organisms, this property is decidedly increased.

5. When transplanted in the human throat, this organism grows in abundance and gives rise to a distinct reaction. It is therefore not a harmless saprophyte.

6. Symbiotic phenomena are manifested by this organism exactly as by Pfeiffer's bacillus.

7. Organisms which have not been differentiated from this bacillus occur in a variety of throat affections, and occasionally in normal throats.

8. The evidence at hand will not permit a definite statement for or against the specificity of this organism for whooping-cough.

9. Granting that it is not specific, its significance as a harmful secondary invader cannot be questioned.

10. This organism was described first by Spengler in 1897, and later by Jochmann, Krause, and others. Undoubtedly many have observed it, but failed to isolate or describe it properly.

I wish to acknowledge my indebtedness to Dr. Hektoen for suggestions and to Drs. Baum and Weaver, attending physicians at Cook County Hospital, for the privilege of utilizing material in the infectious wards. I wish also to thank the internes at Cook County Hospital for their many courtesies, and also numerous physicians who aided me in obtaining material for examination.

EXPLANATION OF PLATE I.

FIG. 1.—Symbiosis—Cluster of colonies of the influenza-like bacillus around the larger staphylococcus colony. Blood-agar plate inoculated directly from pertussis sputum. Magnified 30 times.

FIG. 2.—Influenza-like bacilli from 24 hour blood-agar culture isolated from pertussis sputum. Some of the bacilli show polar staining. Magnified 1,200 diameters.

FIG. 3.—Phagocytosis of influenza-like bacilli in sputum of pertussis patient. Magnified 1,200 diameters.

FIG. 4.—Twenty-four cultures of influenza-like bacilli from pertussis sputum showing marked tendency to thread formation. Magnified 1,200 diameters.

FIG. 5.—Influenza-like bacilli in smear of sputum from case inoculated with these organisms. Magnified 1,200 diameters.

FIG. 6.—Phagocytosis of leucocytes by endothelial cells seen in peritoneal exudate of guinea-pig injected with the influenza-like bacilli. Magnified 1,200 diameters.

PLATE I.

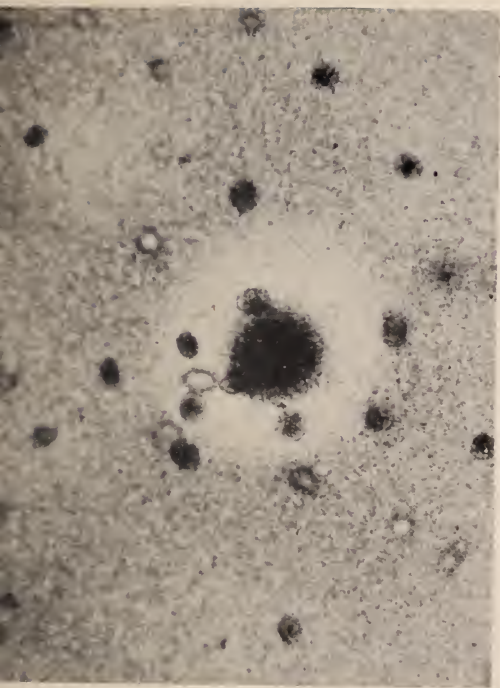


FIG. 1.



FIG. 2.



FIG. 3.

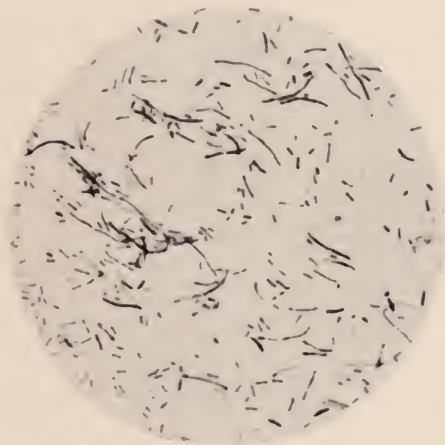


FIG. 4.

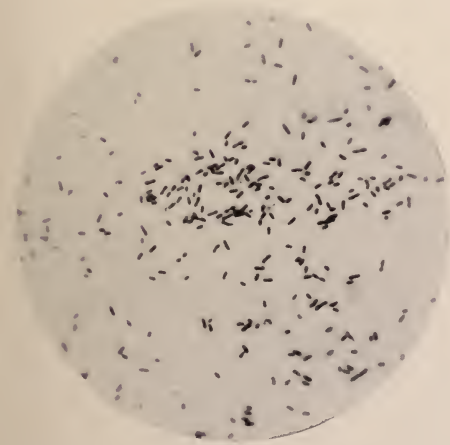


FIG. 5.

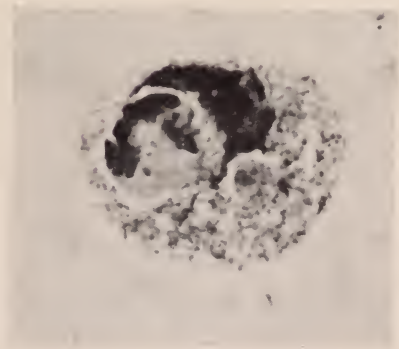


FIG. 6.

STUDIES ON HUMAN PARASITES IN NORTH AMERICA. I. *FILARIA LOA*.*

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CONTENTS.

- I. INTRODUCTION.
 - Origin and Scope of Paper.
 - Data on New Cases in North America.
2. CASES OF *Filaria loa* ON RECORD.
 - List of Genuine Cases.
 - Cases Wrongly Assigned to *Filaria loa*.
3. MORPHOLOGY OF *Filaria loa*.
 - Structure of the Parasite.
 - Life-History.
 - Taxonomy.
 - Geographical Distribution.
4. PATHOLOGY OF *Filaria loa*.
 - Seat of the Parasite.
 - Effect on the Host.
 - Calabar Swellings.
5. CLINICAL DATA.
 - Case of Milroy.
 - Case of Terrin.
6. ANNOTATED BIBLIOGRAPHY OF *Filaria loa*.

I. INTRODUCTION.

IN February, 1902, Dr. W. F. Milroy, of Omaha, brought me a specimen in alcohol which he had just removed from the eye of a patient, and which he believed to belong to the rare and interesting African species, *Filaria loa*. He expressed a desire that I make a more precise examination of the specimen, and that our results be included in a joint communication. The study of this specimen demonstrated that it was in fact *Filaria loa*, and disclosed some interesting features in the anatomy, which, together with Dr. Milroy's clinical observations, were presented before the American Association for the Advancement of Science in 1902. Circumstances have delayed the appearance of the final paper beyond all expectation, and meantime a contribution by Looss (1904) has dealt with

*Received for publication January 2, 1906.

the anatomy of this species so fully as to cover all the points I had worked out. Indeed, the admirable work of this author sets the limits for anatomical studies for many years to come. Accordingly the part of this contribution dealing with the anatomy has been reduced to a brief summary.

Since this preliminary contribution, I have been fortunate enough to secure the data on several other cases in the United States, which have not yet been published, and have had an opportunity to study six other specimens which have been sent me for that purpose. To all the gentlemen who have contributed so generously of their material and have co-operated so unselfishly in securing data on this interesting parasite, I desire to return here my sincere thanks. For these cases I have given the description largely in the precise words of the observer to whom I am indebted for the record. The more extended notes of Dr. Milroy are included in a separate section of this paper.

Through the courtesy of Dr. B. C. Loveland, formerly of Clifton Springs, but now of Syracuse, N. Y., I am able to give the following account of several interesting cases. In two he removed the parasites himself, and one of these, that taken from the eye of Mrs. J., I have been privileged to study this summer. Of the identity of this specimen with *F. loa* there can be no doubt and in the other case also the evidence is strongly in favor of the same interpretation. The location and date lead me to identify the case of Mrs. R. with the one reported by Wilson in 1890 and enrolled as case 31 in my list (p. 47). If this be so, three other specimens were removed from the same host and all these three from the eyelids, additional evidence in favor of assigning this form to *F. loa*. Regarding these cases Dr. Loveland writes as follows:

About 1890 Mrs. R. was under my care, and told me that she was the possessor of one of those worms which would make its appearance at times in the eye and at times come up close under the skin in some other region, where it would produce a sensation of stinging or irritation. I told her to call me at once when it should appear, as she said that it would disappear very quickly into the deeper tissues. She came to my office one evening and told me her worm had come to the surface on her back. And on inspection it appeared not far from the lower angle of her left shoulder blade, where it gave the appearance of a thread drawn in rather crookedly just as close as possible to the cuticle, where it could be felt as well as seen.

I made a quick incision parallel to it in the middle and grasping it with a pair o

small forceps slowly withdrew it as it "let go," so to speak. It was of the type of nematode or round worm, about one and one-half or possibly two inches in length when stretched out, but contracted to much shorter. I had the misfortune to lose it while I was away on a vacation some months later—I think it was a *loa*.

In 1898, while still at Clifton, Dr. Spaulding called me into his office to see something in the eye of Mrs. J., an African missionary patient of his, the like of which he had never seen. I recognized what I thought to be the same worm and secured it at once. This worm I have today mailed you; it, was, so far as I could tell, the same as the one I removed from the back of Mrs. R. in 1890.

She [Mrs. J.] says they are quite common in that part of Africa, Batanga, West Africa, where she was stationed.

She says that her husband and children have all had them. She also says that the worms make sores on the hands or feet and are sometimes captured at those times and places. It is only occasionally that they produce a sore or abscess, and I think that is when, like the Guinea worm, they lay their eggs [embryos] or multiply in a given locality. This last is only hearsay.

These cases came under my care incidentally and have never been reported.

From Dr. C. F. Friend, of Chicago, formerly a medical missionary of the Presbyterian church in West Africa, I have been the recipient of most courteous information regarding a case hitherto unreported. Dr. Friend very kindly sent me the specimen in alcohol together with photomicrographs he made from the living worm and also drawings of the specimen. There is no doubt as to the species which is unquestionably *F. loa*. Regarding this case Dr. Friend says:

This is the only specimen that I have ever removed, and it is that from Mrs. X's eye about four years after her return to America. I am sorry to say that I have lost or misplaced the notes made at that time or I would send them with this letter.

While I have not removed a *loa* from any part of the body other than the eye, yet I have thought that it did travel to other parts, for at different times both Mrs. X. and myself have seen what appeared to be the movement of the worm in different regions of her body. And I have thought that swellings which appear at times on her hands or arm and a time or two on her thigh were caused by the *loa*, as she would have the sensation as of the movement of the worm prior to the swelling, but not always so. In fact at times when we thought we saw the worm in the parts referred to there would be no swelling, and again when I have cut down upon the part when we thought we saw it, we did not find the *loa*. On the other hand, I think Dr. Loveland did remove a *loa* from under the skin of the back of Mrs. R. about 1890. The specimen I am sending you was removed early one morning from under the conjunctiva of the left eye near the outer canthus.

The night before Mrs. X. had complained of a sharp, piercing, pricking sensation, or pain in the eye, which from previous experience she knew to be the movement of the worm, but I could see nothing of it. Upon arising, she could see the worm moving across the eye downward and inward. Mrs. X. thinks that the worm when it appeared in the eye would nearly always, if not always, go out by the way of the inner canthus.

As quickly as possible I prepared the instruments, cocained the eye and with a small pair of locked forceps grasped the worm and the tissues around it. This pair of forceps was then held by an assistant. I then took a lancet and cut down upon the worm and with another pair of forceps grasped it, and after unlocking the other forceps pulled it out, when it wiggled much the same as an earth worm would do under similar circumstances.

One peculiarity that I may mention regarding the action of the worm in this case is that at no time during pregnancy did Mrs. X. feel any movements of the worm. This was noted in two pregnancies prior to the removal of the worm I am sending you, and in two pregnancies in regard to the worm yet in her system.

When cut from the eye the *loa* measured 32 mm. in length.

Through the brief mention of these cases made by Primrose (1903:1264) I became aware of two observations in Toronto that probably concerned *F. loa*. As no account of these cases has been published as yet, I am glad to be able, through the courtesy of two gentlemen to present here the record of the same. It was more than 10 years ago that Dr. F. N. G. Starr showed, at a meeting of the Toronto Pathological Society, a specimen of a filaria he had removed from a patient. The worm was not placed at the time, but subsequent publications on *F. loa* showed its close resemblance to that species, if, indeed, it is not identical with it, as I believe. Since the specimen was lost final evidence cannot be secured. Concerning the case Dr. Starr writes as follows:

The patient, a female, and about 35 years of age, had been for some years a missionary on the west coast of Africa, and because of broken-down health, caused by a series of attacks of fever, she returned. On her way here she had a worm removed.* She presented herself to me with the following story: That at times she would begin to feel an uncomfortable suspicion of burning and fulness in some part of the body and that after a time she could see something crawling under the skin. This would last a few minutes, and then the part where it approached the surface would swell up, be sore for a day or two, and disappear, the amount of swelling depending a good deal upon the location. For example, if near the eyelids there would be very marked swelling. Several times she came to my office, but by the time she reached there the worm had disappeared, and I began to think the life on the West Coast had affected her brain. However, at last she came and I saw the movement under the skin for myself. The appearance was of a thin, white line, drawing itself up, and then projecting one end forward like the movement of a "caterpillar," presently the forward end would begin to disappear, and finally the whole "streak" would disappear from view. The next time, I cut for it, but did so about its middle, and before I could pick up a pair of forceps to grasp it, the worm was out of sight. I then prepared a very sharp scalpel and a pair of fine pointed tissue forceps, and kept

* This specimen of which I have been unable to secure further information was also removed in Canada.

them in readiness, and after repeated attempts, the patient came in. This time the filaria was crawling under the skin of the chest over the manubrium sterni. I cut the skin just behind the forward extremity and made a "grab" into the incision, when the patient assured me I had hold of it for she could feel it squirm. I pulled very cautiously and a threadlike structure came out nearly two inches long, and about the size I should say of a No. 00 catgut suture material. There was never any recurrence of the trouble.

The second case in Canada occurred in the practice of Dr. Frederick Fenton, of Toronto. He removed two specimens at different times from the eyelids of a patient. The specimens were identified as *F. loa*, and although the extended MS. record of the microscopical examination made at the time, which Dr. Fenton was good enough to send for my use, gives nothing which absolutely confirms the diagnosis so far as the species is concerned, yet the details conform fully with the description of *F. loa* and the mention of such items as the well-known cuticular bosses, make the case reasonably certain. The first and larger specimen of which accurate measurements were made was 55 mm. long and 0.5 mm. in diameter; the other was only 45 mm. long. The following data regarding the case are taken directly from correspondence from Dr. Fenton:

Mrs. M., *æt.* 48, was an educated and refined woman, the wife of a missionary. Prior to 1897 she had resided for several years at Batanga, seventy miles inland on the Gaboon River. She first noticed trouble after returning to England in March, 1897. One arm and wrist became greatly swollen and remained so for several months, causing considerable inconvenience owing to degree of swelling, but little if any pain. On subsidence of swelling, part remained "black and blue" for a long time. At times she suffers from fever, pains in the back, and general malaise; there are occasional lancinating pains, as if the worm were cutting its way through the tissues.

I saw her in September, 1898, with Dr. J. L. Davidson, who had tried to remove one and failed from want of assistance. The outline of the worm could be plainly seen lying beneath the skin of the upper eyelid. If touched, and at times when not irritated, it would wiggle through the tissues like a snake. The skin of lid, including the worm, were grasped firmly with a pair of dissecting forceps and an incision made transversely, when the worm was seen lying at the bottom of the wound, looking like a fiddle string or a piece of silkworm gut and was easily picked up and pulled out with a pair of forceps. It rapidly became stiff and hard after removal and was found to be 55 mm. long and 0.5 mm. in diameter, one extremity ending in a hooklike process, while the other is simply rounded off without any apparent thickening. In December of the same year I removed another, 45 mm. in length, from the lower lid, and in the spring of 1899 failed in an attempt to secure another.

On one occasion the worm lay across the center of the field of vision of her left eye for some time, though a careful examination failed to discover it on the anterior surface

of the eye; at that time the worm moved with the movements of the eye, being apparently within the eyeball itself. No ophthalmoscopic examination was made, so there was only the patient's history of the occurrence to suggest the penetration of the eyeball.

These specimens were shown before the Toronto Pathological Society, and a brief history given, but no paper has been published concerning them. I saw this patient again in May, 1899, and up to that time she had had no further trouble.

The largest group of specimens I was privileged to examine came to me through the courtesy of Dr. J. H. Murphy and Dr. D. T. Vail, of Cincinnati, Ohio. In addition to several fragments belonging probably to two worms, there were two perfect specimens of a female *F. loa* in alcohol and one specimen in balsam probably entire, although both ends of the latter worm were badly mutilated or shrunken in mounting and so imperfectly cleared that it was impossible to determine the sex or the character of these parts of the body. The cuticular bosses which were so well described and figured by Blanchard (1889) for *F. loa* are distinctly visible and the general appearance of the body, in comparison with other unmistakable specimens of *F. loa*, leaves little doubt that this worm belongs to the species under consideration. The precise determination of this specimen is all the more important since it is the one removed by Dr. L. from his wife's breast. He extracted one of these worms from the skin overlying the sterno-cleido-mastoid muscle, and another from her left breast. The entire specimen in alcohol bears his name also on the label and is no doubt the other worm noted. It is a perfect specimen of a female *F. loa*. I think this is the first instance in which a supposed *F. loa* removed in life from any other part of the body than the vicinity of the eye has fallen into the hands of a helminthologist for careful examination and determination. In view of the very large number of filariæ already reported from Africa, even though the fauna is necessarily most imperfectly known, the reports of the extraction of the *loa* from other parts of the body than the eye have been received with some caution by helminthologists. This is clearly shown by the silence of Manson, Blanchard, and other authorities on this point, even though they cite in connection with some cases in the eye the popular opinion that such worms occur elsewhere in the body. In the present case we have the best of evidence, since the specimens in question were removed by a medical man,

and on account of the importance of the matter I have subjected them to most careful scrutiny. While one specimen is not in sufficiently good condition to render an absolute decision possible, there can be no doubt as to the systematic position of the other. Accordingly it may now be affirmed that the *F. loa* does make its appearance near the surface in other parts of the body than the eye. Since Dr. Vail has in preparation a paper, read before the American Academy of Ophthalmology and Oto-Laryngology at Buffalo in September, 1905, I forbear to trench further upon his field, and refer to his paper for further details regarding these cases and for a discussion of the clinical factors.

2. CASES OF *FILARIA LOA* ON RECORD.

Many authors have assembled the earlier records of this parasite, but in general the lists given have been inaccurate and imperfect. The series given by Blanchard (1899) is admirable in manner of treatment and is the most complete. It includes 25 previous cases and one new one. The method employed of listing all records quoted from a given paper as one case under the name of the author seems to me undesirable, since it does not distinguish between the account of a single chance specimen and more extended observation. Here each case includes the history of only a single host, so far as this could be fixed, even though two or more parasites were removed from the one individual. If this method be criticised as incomplete, one can only reply that it is impossible to determine whether the multiple infection took place at a single time or through repeated introduction of the parasite. Only the positive demonstration of the latter condition would justify the interpretation of the numerous parasites as separate cases of the disease. I have departed from this rule twice when the time interval was such as to justify regarding the later record as a new case. So far as possible each case record includes the name, date, and place of observation; the sex, age, and nationality of the person infected; the number and sex of the worms; a statement regarding their removal, if accomplished; the probable place and time of infection; and finally the place and date of publication. In some cases only a limited amount of data are given by the original

recorder, and in many instances certain of these desiderata are lacking.

By no means all of the cases of which we have reasonably good information are included in the list, since some of the records, though distinct, are not definite enough to enumerate exactly in such a series. Thus Guyot (1805) speaks of several other individuals, on the coast of Angola; Wilson's patient says (Wilson, 1890) the disease is common among natives, and all the missionaries of that station, Benita, near Gaboon, have them; Robertson's patient had seen such cases in the eyes of natives; Roth (1896) says his patient informed him that a number of people in her village complained of the same disease; while Miss Kingsley, the well-known African traveler, speaks of these filariæ as abundant and fairly common in different regions of the west coast of Africa. Such evidence might be multiplied concerning this part of the world.

Not all cases are equally clearly established. I have followed the general custom of previous authors in including cases in which the identity of the parasite has not been finally demonstrated. Indeed, were one to demand precise identification, all the earlier cases and many of the later ones must be thrown out. Again, other species have been reported from the eye of man, and some of those doubtfully attributed to *F. loa* in this list may belong to such species. In such cases the geographical location of the case or the past record of the infected person are of importance in determining the probable species of filaria represented. Even thus no case has been included in this list except the weight of evidence was strongly in favor of the interpretation given. Under this treatment the total number listed becomes 97, from the record of Mongin, published in 1770, to those of the current year (1905), a time interval of 135 years. About two-thirds fall within the last 20 years, and half the total number have been published within the 10 years from 1896 to date.

The matter of the earliest record calls for a word of comment. Pigafetta (1525) has been cited by Guyon (1864), Manson, Moniez (1896), and Blanchard (1886, 1899) as evidence of the occurrence of *Filaria loa* in Africa in the 16th century. This claim is based upon a plate, one figure of which is interpreted by these authors

as illustrating the removal of an eyeworm. It appears that this plate does not belong to Pigafetta's works, but to Lindschoten's; and even here it is not found in the original edition (1596), but occurs first in the De Bry reprint where it was probably inserted by the publisher. I have discussed the matter in detail elsewhere (Ward, 1905). The region described by Lindschoten lies in the Persian Gulf, and not in the Congo territory, where Guyon *et alii* located the account. It is thus well within the range of *Dracunculus medinensis*, but far removed from the habitat of *Filaria loa*. Furthermore the text makes no mention of infected eyes, but speaks of "worms in the legs" of the natives, which again accords with the Guinea worm. Hence the interpretation placed upon the plate must be rejected, and if, indeed, the plate itself has any standing as evidence, it concerns the Guinea worm rather than *F. loa*. This reference must accordingly be eliminated from discussions of the latter species. It is not listed here among the cases of *F. loa*, which I have collected, verified, and arranged as follows:

LIST OF GENUINE CASES.

1. Mongin, at St. Domingo, in 1770, records the extraction of one worm from between the conjunctiva and albuginea of a negress.
2. Bajon, at Cayenne, in 1768, removed a worm from below the conjunctiva of a negress eight years old; this case was first published in 1777 together with the following
3. Also at Cayenne, in 1771, Bajon observed in an older negress such a worm moving across the eye between conjunctiva and cornea, but was not allowed to remove it.
4. Mercier, at St. Domingo, in 1771, extracted a worm from beneath the cornea of a negress.
5. The same authority, in 1774, removed from a negro a worm which lay above the cornea. The record of Cases 4 and 5 was published by Arrachart in 1805.
6. Arrachart notes that in 1795 Mlle. L. Fraise, creole, born in St. Domingo, assured him that her brother had several times such worms in his eyes at the age of three to five years; they were successfully extracted. She also adds that young negroes were often attacked in this way. This striking note seems to have been overlooked by students of the subject. The direct implication that the child was born in St. Domingo would indicate the existence there at that time of a center of infection for *F. loa*.
7. The French naval surgeon, Guyot, made several voyages to the coast of Angola. On one occasion, examining closely the eye of a negress, he saw what seemed to be a varicose vein in the conjunctiva, but when he touched it with the point of a lancet the object disappeared. It appeared several times in the same patient at irregular intervals, and he thought that between times the worm retired to the posterior region of the orbit. He recorded the native name of *loa*, the common occurrence of the malady, the irregular appearances of the worm in the eye, and the inefficacy of all medication. The case was first published in Arrachart, 1805, together with the following.

8-12. In 1777 Guyot made a new voyage to the coast of Angola. He observed again this verminous ophthalmia among the negroes of the Congo, and in two cases out of five succeeded in removing the worms. The account of these cases was first published by Arrachart (1805: 228, Observations 7ff.) and later by Rayer (1843). Guyot was the first to view this species as different from the Guinea worm. He says: "Je ne crois pas que ces vers soient de l'espèce du dragoneau, car ils sont très blancs, plus dur et moins longs à proportion. Je n'ai jamais vu ce ver se faire jour de lui-même. Pendant sept voyages que j'ai fait à la côte d'Angola, je n'ai vu aucun nègre attaqué du dragoneau. Plusieurs chirurgiens qui ont navigué sur ces côtes m'ont assuré n'en avoir jamais vu."

13. M. de Lassus, army health officer of St. Domingo, removed a worm from the eye of a negro. The case is chronicled by Larry, 1812.

14. In 1828 a worm was seen in the orbit of a negress, recently arrived as a slave from Africa at Monpox, a village on the banks of the Magdalena River in United States of Colombia. This observation is attributed unmistakably by the original text to Clot-Bey, a French surgeon, well known for his work in Egypt about that date. The French authors agree in pronouncing this authorship an error and in substituting the name of Roulin. I have found neither explanation nor reference to Roulin or his works.

15. Dr. Blott, a physician on Martinique, in 1837 removed two filariæ from the eye of a young negress who had come from the African coast. One was sent to Guyon and Blainville, and described by the former (Guyon, 1838).

16, 17. Loney, an English naval surgeon, in April and June, 1842, extracted moving worms from beneath the conjunctiva of two natives on the west coast of Africa. He reported these cases together in 1844.

18. Lallemand excised a worm from the eye of a negro in Rio de Janeiro, and in 1844 published a description of the case.

19. In Christovó, José dos Santos removed a worm from the orbit of a Mina negress. Sigaud witnessed the operation and reported it in 1844.

20. Lestrille in 1854 removed a worm from the eye of a negro at Gaboon; his description of the case was published by Gervais et Van Beneden (1859).

21. Mitchell saw such a worm in 1845 at Trinidad. The host, a young negress, had come from the west coast of Africa in 1834; the worm made its first appearance in the left eye in 1837, again in 1841. The specimen Mitchell saw was presumably at least 11 years old, although he infers wrongly that the various reports necessarily concern the same individual parasite. According to tradition one had been seen in a family in Antigua 60 years before. Mitchell reported his case in 1859.

22. In 1864 Guyon reported another specimen removed by a marine surgeon from a negro in Gaboon. Part of this worm remained entangled in the deeper tissues of the orbit.

23. In March, 1868, Dr. Maurel, at Gaboon, removed a worm from the eye of a native. Trucy (1873) reported the case as Observation 3, in a paper on the Guinea worm.

24. Rev. Dr. Nassau, a missionary in Gaboon, sent in 1876 to Dr. Morton, a surgeon in Philadelphia, a *loa* taken from the eye of a native woman. The worm was examined by Leidy, whose brief description and the account of Dr. Nassau, which also includes Cases 25 and 26, were published by Morton (1877).

25. Rev. Dr. Nassau records that while he has never had the worm in his eye, he has yet seen it moving beneath the skin of his fingers. In Gaboon the worm shows itself at various points of the body of the host, in the fingers and eyelids, as well as under the conjunctiva. He has seen the worms both in his own fingers and in those of other persons. The effort to extract one specimen from his eyelid failed by virtue of the activity of the worm. Though evidently incomplete, this observation furnishes the first suggestion that the parasite is not exclusively confined to the region of the eyes.

26. An English trader, Captain Stone, living on the Ogooué, had one removed from his eye by a native using a thorn as a needle. The case is quoted from a letter by Dr. Nassau in Morton, 1877.

27. Dr. Bachelor, of Gaboon, extracted a specimen from the eye of a native young man. It was on the iris beneath the sclera. This was the first perfect specimen sent to the United States. The case is reported in his letter (Bachelor, 1880).

28. Dr. Bachelor reported a year later (1881) the case of a white woman, a missionary near Gaboon, from whom at different times three such worms were removed. He also confirms the record (Case 25) that Dr. Nassau, who was frequently affected, "had one in the areolar tissue between the thumb and index finger."

29. Dr. Falkenstein sent Leuckart from the Loango coast a specimen of this worm from the eye of a European, which was determined and reported as a species clearly distinct from the Guinea worm (Leuckart, 1881).

30. Dr. Lota, a French physician in Gaboon, experienced conjunctivitis after his return to France, and on careful examination saw such a worm beneath the conjunctiva. He noted its movements and demonstrated the case to several colleagues; but the worm disappeared before removal. His eyesight was not impaired. The case is chronicled by Terrin, 1884.

31. Mrs. —, missionary at Benita, near Gaboon, had at intervals felt and seen such worms. She had one removed in February, 1889, at Basel, Switzerland, from the left upper eyelid; one in November, 1889, at Bridgeport, Conn., from the right upper eyelid; one in February, 1890, at Clifton Springs, N. Y., from beneath the skin of the back; and in July, 1890, one broke in removing it from the upper eyelid. She says the worm is common in Benita and all the natives have them, and the author adds: "So far as I have been able to obtain evidence from the missionaries themselves, the filariæ are more common in the cellular tissue than in the eyeball. From the literature we should infer the opposite." The worm was removed and the case reported in 1890 by Dr. F. M. Wilson of Bridgeport, Conn.

32. One other missionary at Benita had such worms removed. The fact is chronicled by Wilson (1890) on the direct testimony of his patient of Case 31.

33. An infant negress from the Congo had a worm in the anterior chamber of the eye. It was reported by Coppez (1894), van Duyse (1895), Gauthier (1895), and Lacompte (1894). When extracted by the latter, it was dead.

34. An English woman who had lived eight years in Old Calabar felt the parasite a month after her return to England, but later thought it had disappeared, as one was passed *per rectum*. Eight months after her return a male was removed from one eye by Dr. Robertson and reported by him (1894, 1895). From the same patient he removed subsequently (1895: 162) a female worm. Further history of this patient is recorded in Case 73.

35. A woman who lived at Old Calabar from 1860 to 1863 had suffered while there from a worm in the eye. After her return she had a *loa* removed in 1875 and a second in 1876. The case is recorded by Robertson (1894, 1895).

36, 37. Dr. Thompstone, of Opobo, in Nigeria, described two cases of *loa* in natives. One was in the lower eyelid, the other beneath the conjunctiva. He was not able to remove either worm. These data were published by Robertson (1894, 1895).

38. In a woman at the same mission with Case 33 the worm was seen to pass from one eye to the other over the bridge of the nose. It was not removed. The case is recorded in Robertson (1895).

39. A missionary in Old Calabar had a *loa* which showed itself at irregular intervals for about 15 years and then disappeared without having been removed. Robertson (1895) gives the record of the case.

40. Dr. J. R. Logan, of Liverpool, removed a male *loa* from the eyelid of a patient. The blood of this patient was examined for filariæ, but held none. This worm was examined and described by Manson (Robertson, 1895). No further data are given.

41. A female *F. loa* was taken by a merchant from the eye of a negro at Cayo (French Congo) and sent to Berlin. The case was recorded and discussed by Hirschberg (1895).

42. In 1895 Dr. Saemisch extracted a *loa* from the eye of a Russian marine officer who had been in Fernando Po from 1886 to 1891, and in Gaboon, Kamerun, and the Gold Coast from 1882 to 1885. The parasite was carefully described by Ludwig (Ludwig and Saemisch, 1895).

43. In July, 1895, Roth observed an extremely active *loa* in the eyelid and just above in a Jackrie girl at Warri, on the coast of Nigeria. He failed in the effort to remove it.

44, 45. Later the same author (Roth, 1896) observed similar worms in two other natives without being able to extract them. He believes they pass out through the nasal duct. In spite of their frequency a reward failed to secure specimens.

46. In 1893 Barrett removed a worm from the eye of a young white man who had lived on the Gold Coast but had left there four years before and since then had resided in Melbourne; it was the first specimen removed in Australia. The worm was examined by Professor Dendy and determined as *Filaria oculi humani*. Barrett reported the case in 1896.

47-49. In three natives of Kamerun Dr. Plehn observed specimens of *loa* in the eye. He attributed to the worm also the variable cutaneous inflammations found on the west coast of Africa, and discussed them at length (Plehn, 1898).

50. In an English official Plehn also knew of a case, although he did not see the worm himself. According to the natives this worm occurs also in the eye in goats and sheep. He records these facts in the paper cited above (Plehn, 1898).

51. A French missionary who spent 1894-96 near Ogooué in French Congo, was relieved of a male *F. loa* by Dr. Bernard in 1898 at Paris. Bernard describes the case (1898) and sent the specimen to Blanchard for study. This was, in fact, the second specimen taken from the same host; the first specimen was described later. (See Case 52.)

52. Dr. Leneveu removed a female *loa* from the same host in August, 1897. The case is recorded by Blanchard (1899), who also gives an extended account of the anatomy of the two specimens.

53. Manson had a negro patient under his care in whose blood *F. diurna* abounded. When a lad he had a *loa* in his eye. The case is recorded in Manson (1900: 560).

54. A lady long resident in Old Calabar had a *loa* extracted from under the skin over the right clavicle. She informed Manson (cf. Manson, 1900: 562) that if rubbing

or scratching is not indulged in when a *loa* approaches the surface there will be no swelling, and that Calabar swellings are produced by the rubbing solicited by the irritation caused by *F. loa*.

55. Annett, Dutton and Elliott (1901) record that at Bonney they were fortunate enough to obtain a single female of this species for their collection. Since nothing is said regarding host and location, it is fair to assume its removal from the usual place, the eye of man.

56. The same authors received a female parasite taken from the eye of a Kroo boy by Dr. A. H. Hanley, medical officer at Opobo. In the blood of the host were embryos most similar to Manson's *F. diurna*.

57. Dr. A. H. Hanley also sent a male *F. loa* from the eye of a Kroo boy whose blood had no embryos at all. This case is recorded by Annett, Dutton and Elliott, 1901.

58. In 1902 Dr. Milroy removed from a man who had been a missionary in Batanga a male *F. loa*. It was first observed in 1899. The case was first published by Ward (1902), but the full account by Dr. Milroy is found in this paper.

59. Dr. Rennes removed two specimens of *F. loa* from a European in Sierra Leone, where no previous case has been noted. The patient had been living in the Congo and had been in Sherboro only one year. One worm was removed from the eyelid and the other from the loose skin of the penis. The blood of the patient was swarming with embryos. The case is recorded by Prout, 1902.

60, 61. Dr. Thompstone removed two males and two females from natives of Opobo, Nigeria, and sent them to Dr. Manson of London. They were described by Ozzard, 1903; no data are given regarding the hosts, but they were probably natives.

62-67. At the mission station of Yakusu, near Stanley Falls, upper Congo River, Mr. S. S. found *F. loa* very common among natives. He saw at least six cases. The record was published by Manson, 1903.

68. Dr. Frederick Fenton, of Toronto, Canada, removed two worms from the eyelid of a patient in September and December, 1898, and failed in 1899 in the effort to secure a third. The case was presented to the Toronto Pathological Society but not published. It was noted briefly by Primrose (1903) and is published in full in the present paper (p. 41).

69. Dr. F. N. G. Starr, of Toronto, Canada, removed a filaria, probably *F. loa*, from a female patient who had been a missionary on the west coast of Africa and had returned to Canada on account of ill health. The worm was taken from the skin above the manubrium sterni. The specimen was shown at a meeting of the Toronto Pathological Society about 10 years ago. The case was briefly noted by Primrose (1903) and its data appear in full in the present paper (p. 40). Dr. Starr's observations are apparently the first made by a physician on the movement of such a parasite in the body outside of the region of the eye.

70. Dr. Habershon (1904) records from Yakusu, on the Congo River, that in Mr. K. S., afflicted with Calabar swellings, a *loa* was seen to cross the conjunctiva.

71. Habershon (1904) also adds that the same conditions were observed in a native.

72. Dr. D. Argyll Robertson says that his patient suffered from Calabar swellings and noticed worms (*F. loa*) in her side, left shoulder, under the skin of both hands, under the abdominal wall, and in her right breast. The parasites were successfully extracted from the last two situations. The record was published in Habershon, 1904.

73. Dr. Robertson also records the case of another English woman from Old Calabar in whom *F. loa* was seen under the conjunctiva while she herself noted them under

the skin of hands, wrists, breast, face, and scalp. Four attempts to remove them from under the skin of the nose, hand, and arm, failed. He says further that there is no doubt that in many cases several worms are present in the same host. The record was published by Habershon, 1904.

74. A young French girl who had stayed several years at Libreville (Congo) was taken in 1902 with painful localized edemas of both hands and wrists, occasionally of legs, associated with some rigidity and loss of power. A white worm about the size and length of an ordinary pin was seen beneath the ocular conjunctiva, reappearing later beneath the skin of the eyelids of both eyes, of both forearms, and finally under the frenum of the tongue. Attempts to remove the worm failed. She returned to France in 1903 and a *loa* was extracted from the eye in January, 1904. An intense eosinophilia was noted in 1903, and, though subject to fluctuations, continued after the removal of the worm. Probably other parasites also were present. The case is recorded by Wurtz and Clerc, 1904, 1905, and Kerr, 1904.

75. Rev. S. O. K., from Yakusu, on Upper Congo, where he had been for three years, returned to England in January, 1904. Localized swellings, chiefly on the left forearm, first appeared after one year in Yakusu. Blood examinations showed microfilariae with diurnal periodicity well marked, hence diagnosed as *F. diurna*. The case was sent by Dr. Habershon to Sir Patrick Manson and described by Kerr (1904).

76. In a European who suffered from these transient swellings there was also a *Filaria loa* present, and in the blood numerous embryo filariae which could not be distinguished from *F. diurna*. The case was observed by Dr. Hanley, of Old Calabar, and published by Kerr (1904).

77. From a native of Old Calabar a *F. loa* was removed and found to be full of sheathed embryos, indistinguishable from *F. diurna*, which were also found in the blood. No mention is made of swellings in this case by Dr. Hanley, whose account was published by Kerr (1904).

78. At an autopsy of a Congo negro who died in Paris of sleeping sickness, Penel (1904: 207) found more than 30 adults scattered through the superficial connective tissue of the four appendages, and despite most careful search not a single specimen could be discovered in the neck, face, or region of the eye. Full data are found in Penel (1905a: 127).

79. In 1904 Looss published an account of the structure of *F. loa* based on three specimens from the Gold Coast; their source is unknown. They represent at least one case of human infection with this parasite.

80. At an autopsy of a native in Kassai, Brumpt found among other specimens, encysted and so completely calcified as to be unrecognizable, a fragment of a filaria encysted in the heart, which on return to France and comparison he identified as *F. loa*. It was a female and contained embryos identical with those in the blood of the same host. The case is recorded in Brumpt, 1904.

80a. Texier has recorded another case (*vide* Penel, 1905a: 67, 82). The original was not accessible.

81. A specimen 60 mm. long was taken from beneath the conjunctiva of a man who had lived in Kamerun from 1897 to 1898 and since then in Germany. There was no intimation of the parasite until the day before removal. The case is recorded by Pick, 1905.

82. Dr. Hans Ziemann records (1905) that he had in his earlier service one case of *F. loa*. The host was presumably a native and the locality probably the same as that given for the following record.

83-86. The same author records the occurrence of four cases in his later service. Apparently he was stationed at Duala, Kamerun.

86a, b. Lippert writes (Vail, 1905) "since my return [to Kamerun] I have taken out numbers galore." He mentions two cases in detail. One taken from the upper eyelid was "over three inches long," the other motionless under the conjunctiva appeared nodular and was adherent to the surrounding tissue.

NEW CASES.

87. Mr. K. observed that on one occasion, when a Calabar swelling upon the back of a woman's hand was rubbed, such a worm was seen to emerge from the tumefaction and make its way across the metacarpo-phalanged articulation, from which location it was extracted. These data are recorded by Milroy in the present paper (p. 75).

88. In 1890 Dr. B. C. Loveland removed a *loa* from the skin above the lower angle of the left scapula of Mrs. R., formerly a missionary near Batanga, West Africa. Recorded in the present paper (p. 38).

89. In 1898 Dr. Loveland extracted a *loa* from the eye of Mrs. J., also a returned missionary from Batanga, West Africa. The specimen I have described in this paper (p. 39) and the case is recorded here also (p. 39).

90-92. On the evidence of Mrs. J., her husband and children have all had the same parasite. The fact is recorded by Dr. Loveland in this paper (p. 39).

93. Dr. C. F. Friend removed a *loa* from the eye of Mrs. X., formerly a missionary in West Africa, about four years after her return to America. This specimen is described in this paper (p. 39) and the data on the case are also recorded herein (p. 39).

94. The case of Dr. D. T. Vail, of Cincinnati, Ohio, briefly referred to in the preceding pages (p. 42) and reported at length before the Buffalo meeting of the American Academy of Ophthalmology and Oto-Laryngology.

CASES WRONGLY ASSIGNED TO *F. LOA*.

It is no matter of conjecture that other species of filaria than *F. loa* do occur in the human eye. In Italy for example Addario (1885) observed in the eye of man a nematode which he named *F. conjunctivae*. Later Grassi (1887) published an extended description of the same form to which he gave the name *F. inermis*. He also discussed the cases of its occurrence in man, and showed it to be a normal parasite of the horse and ass that as an erratic parasite occurs at times in the human eye. In spite of a certain similarity in general character, its differentiation from *F. loa* is not a matter of any difficulty in case a precise examination is made of the specimen in question. However, when no such examination is recorded the area of geographical distribution becomes determinative in general, and cases with insufficient data occurring within the range of this or a similar species will be referred to it by preference rather than to *F. loa*.* Thus the cases from Italy in so

*Reciprocally it is just to assign to *F. loa* such cases as that of Maurel (Trucy, 1873) since the parasite was removed at Gaboon where the *loa* is common, while it is beyond the range of the Guinea worm, to which the case is referred by the author.

far as they are not errors in observation are naturally assigned to *F. conjunctivae* in the absence of more precise information as to the actual species concerned.

In similar fashion the case of Drake (1894) from Madras, India, is regarded by Blanchard as belonging most probably to *F. equina*, a common parasite of the horse and ass in that region and known in such hosts to make occasional incursions into the eye. The case of Neve (1895), also from India, in which the parasite was designated specifically as *F. loa* appears to me to be undoubtedly an error in determination and to concern rather the species of *F. equina*. I was unable to consult a copy of the paper by Macnamara (1863) which, to judge from the title, refers to cases assigned to the species *F. papillosa* (= *F. equina*) as occurring both in man and in the horse in India.

It is of great interest to note that in North America is found a species which occurs at times in the eyes of the horse. Such cases are recorded for Canada by Sermon (1872), and for Pennsylvania by Turnbull (1878). In spite of the designation of the parasite in the first case as *F. oculi*, much used for *F. loa* by medical authorities, we are justified in attributing the case to some other species since the patient was a bay mare. Now the occurrence in this territory of a filaria in the eye of the horse necessarily casts a shadow of doubt upon cases in man in which the supposed *F. loa* was not carefully examined since it has been noted species of similar habit in Italy and India occur at times also in the human eye. It is indeed altogether likely that cases will occur in this country in which the horse parasite will, as an erratic, invade the eye of man.

In view of these facts one would be justified in expressing doubt as to the correctness of certain cases generally listed with *F. loa*. In particular the cases of Lallemant (No. 18) and dos Santos (No. 19) from Brazil, may justly be questioned. To be sure both were originally regarded as cases of the Guinea worm, and only by later authors have they been interpreted as *F. loa* by virtue of their occurrence in the eye. While I am inclined to regard this habit as sufficient reason for rejecting the original determination, it should be confessed there is some ground for doubting the assignment of

the worm to the species *F. loa*. The cases are unique in Brazil, and there is no evidence that the hosts, although of negro blood, were recent importations from Africa. Now while there attaches some doubt to all cases in which a positive determination of the specimen was not made, yet when the history of the host shows recent importation from Africa as in many of those reported from the West Indies the uncertainty is very slight. When the case history is not so clear, the possibility of a chance infection with some form indigenous to the region is not definitely excluded. In other words, should future study show the presence in Brazil of some species such as is *F. conjunctivae* in Italy, the cases so definitely assigned by previous authors to *F. loa* would necessarily be withdrawn from the list. That such species are found in Brazil one cannot doubt in view of the investigations of Daniels on Carib Indians of British Guiana, and of Magalhães on various hosts in Brazil itself. That any of these species occur in the eye I have not yet found on record.

Other cases referred to by some authors as *F. loa* or listed in probable connection with that species should be stricken from the list on other grounds. One of the most difficult to explain satisfactorily is the case of Barkan (1876). The patient, an Australian, was operated upon in San Francisco for an eyeworm and the specimen which was submitted to Dr. H. Knapp of New York was pronounced upon microscopical examination to be "*Filaria medinensis*."* There was no evidence that the patient had ever been in any region where either the species noted, or *F. loa*, with which it might easily be confused, is endemic. Consequently I am inclined to believe that the form was an Australian filaria, normally occurring in some other host, but in this case appearing in man as an erratic.

For various reasons noted in the bibliography one is not justified in assigning to *F. loa* the cases of De Mets (1876), Kuhnt (1892), and von Nordmann (1832). Although in all three cases nematodes were actually demonstrated, they are so unlike *F. loa* that their distinctness from this species can hardly be questioned.

* Dr. Knapp kindly informs me that, at my request, he has made every effort to trace the specimen and that he fears it has been destroyed

Still less connection with *F. loa* have the cases of Eversbusch, Fano, Malgat, Piccirilli, Quadri and Schöler. The specimens of Piccirilli were observed in the anterior chamber of the eye, the worm seen by Schöler was in the lens, the other objects were all located in the vitreous body. All of these cases agree in that the supposed filaria was observed living in the eye by means of the ophthalmoscope. Such evidence is exceedingly questionable; in most instances manifests its weakness on close examination of the record, and in one case at least (Fano) demonstrated its insufficiency by a second examination eight years later than the original, which disclosed only trivial changes in the position and character of the object. One may also infer that in one case at least (Eversbusch) the author became convinced of the insufficiency of his evidence, since only a brief preliminary communication has appeared and the extended report which was promised therein has not been published. Subsequent authors have not hesitated to pronounce these observations erroneous, and to maintain that, in fact, the authors mentioned had to do with cases of a persistent hyaloid artery in which this vessel exhibited a peculiar wormlike form, while the supposed twistings of the filaria were only the results of vascular pulsations or of movements in the vitreous humor. The explanation accords fully with the original records as I can distinctly affirm after a careful study of them, and indeed elucidates certain points otherwise inexplicable, such as the statement of Fano (1868), that the head of the worm remained constantly fixed at a given point while the body turned and twisted about. Since I have been unable to trace the references to Chiraltz and to Santos Fernandez, it is impossible to say whether these cases of a filaria in the vitreous humor are to be explained on the same basis or whether a filaria was actually present.

* Quite recently Nakaizumi (1903) has reported a case of a filaria in the vitreous humor which he regarded as an immature *F. loa*. This conclusion appears entirely inadmissible, even though one rejects the opposite extreme of interpreting this case, like those just discussed, as an abnormal structure belonging to the eye itself rather than as a filaria. The history of the case gives no evidence that the patient had ever been in a region where *F. loa* was

endemic, and consequently where an infection with this species could have taken place. Furthermore no evidence is adduced to indicate the specific character of the filaria observed. If then one grants that the object actually was a worm belonging to the genus *Filaria*, it is certain that it could not have been *F. loa*, but was some species indigenous to northern Europe, and probably *F. conjunctivae* or *F. equina*. The habit of the patient, who is said to have enjoyed half-roasted horse flesh, may indicate an infection with a young *F. equina*. It is exceedingly unfortunate that the literature of science should be loaded down with such incomplete observations, and this is entirely unnecessary when the observations are made at such a time and place as that in question, where accurate data regarding these species were easily obtainable.

For reasons given *in extenso* elsewhere (Ward, 1905) and already noted in the present paper, we must reject the classic reference to Pigafetta, more correctly Lindschoten, as the earliest authority to record a case of *F. loa*.

3. MORPHOLOGY OF *FILARIA LOA*.

STRUCTURE OF THE PARASITE.

The appearance of the admirable account of Looss (1904) makes any extended consideration of this topic superfluous. Only those points are noted which are peculiar to the specimens of this paper.

In all I studied carefully three males, those removed by Drs. Friend, Loveland, and Milroy, and have examined two others, probably males of *F. loa*, sent me by Dr. Vail. I have had only one female, an alcoholic specimen removed by Dr. Lippert and sent me by Dr. Vail.

One male from Dr. Vail measured about 16 mm. in length, though the shrunken condition of both ends makes this measurement only approximate; the other male of this collection was not complete. The male *loa* in alcohol from Dr. Friend measured 25 mm. in length,* and the specimen from Dr. Loveland, which was mounted in balsam and appeared somewhat shrunken, was about 22 mm. in length.

In no one of these males was the tip of the tail as straight as figured by Looss, but curved distinctly though only gradually.

* Dr. Friend gives the length of this specimen living as 32 mm.

From my original notes on the specimen of Dr. Milroy I excerpt the following:

The specimen measured approximately 28 mm. in length and in alcohol was of a clear brown color with distinctly marked lateral lines. The slightly reflexed posterior end and projecting spicules showed it to be a male. A more careful examination of this region disclosed the four pairs of large circumanal papillæ characteristic of *F. loa*. One important feature was noted in this connection. These papillæ do not constitute four bilateral pairs, but rather a left and right series of four each in which the individual papillæ alternate with each other, those of the left side being the more anterior, while those on the right are more closely crowded together. The anterior papilla is also the largest in each series and the size decreases regularly posteriad.

This asymmetrical arrangement originally described by Looss is not an abnormality in the specimen he studied; and I can confirm his view that it is a general characteristic. At least it is actually present in the three males I examined, and will no doubt be found on more extended examination to be universal.

Posterior to these large papillæ lie first a symmetrical pair of small papillæ, and then almost at the tip of the body, according to Looss, a minute pair, also symmetrically placed. The latter I was unable to find.

In the specimen received from Dr Loveland the spicules could be most clearly seen; their length was 104 μ and 180 μ , measurements which accord closely with those given by Looss. Further than this my observations, though in some respects less complete, merely confirm the anatomical descriptions given by Looss. It is important to call attention to the results of a comparison of measurements of *F. loa* given by various authors, and since only relatively few have given sufficient data for the determination of the sex of the parasites, the figures available are much more limited than the number of cases.

According to various records the measured length of the male is 22 mm. (Blanchard), 23 mm. (Looss), 25-30 mm. (Manson), 30 and 35 mm. (Ozzard), and 16, 22, and 25 mm. (Ward). It is noteworthy that the female varies more widely; the measurements given

are 20 mm. (Blanchard), 50 mm. (Annett, Dutton, and Elliott), 52 mm. (Looss), 27 mm. (Leuckart), 41 mm. (Ludwig), 32.5 mm. (Manson), 50 and 55 mm. (Ozzard). Blanchard notes that his specimen was still young, and yet even that of Looss was far from having attained the size of Maurel's specimen, which measured 70 mm. and which, from its extreme length, we are justified in regarding as a female.* The specimen reported by Brumpt (1904) measured 60 mm., and yet it was only part of a female, both head and tail being lacking. The specimens of the female taken from the eye are thus usually, if not always, only partly grown. How much they fall short of full size can only be determined by the records of specimens taken from postmortem examinations, which have settled down in deeper tissues and are found to be producing embryos.†

LIFE-HISTORY.

Concerning the life-history of *F. loa* only meager facts are at hand, and yet they are so clearly related that one may sketch the main course of development with great probability. Manson (1893) was the first to suggest that the blood-inhabiting embryo called *F. diurna* was the young form of this species. The agreement in the geographic distribution of the two forms, the certainty that in the infected region the embryonic stage of *F. loa* must be common, and absence of any other microfilaria made the genetic connection of the two almost an established fact. Yet the negative results of blood examination in several cases which harbored *F. loa*, especially that of Robertson (1895) from which both male and female *F. loa* had been removed, served to cast doubt upon the view. Such doubt was distinctly unjustified since, as I have pointed out, the forms extracted from the eye have been consistently immature and have been removed before the female has begun the production of embryos.

These conditions of probable slow development and of immaturity when in the eye agree well with known facts from related species of *Filaria* in other animals. Thus *F. equina*, a common

* Lippert reports very recently (Vail, 1905) that he removed from the upper eyelid a *loa* "over three inches [72 mm.] in length."

† If the record of Guyon (1864) that his specimen was 15 cm. long does not rest on an error in transcribing or printing, it represents a much larger and hence more nearly full-grown female than any yet recorded. Ludwig has already shown that this case in all probability concerns *Filaria loa* (cf. Ludwig und Saemisch, 1895: 737).

parasite of the horse and ass, which occurs at times in the eye of the host, is found there in the semi-adult form which is also an active migrant. *F. labiata-papillosa* of deer and cattle appears, when immature, in the eye; and in a large number of cases, immature nematodes of unrecognized species, often belonging to the genus *Filaria*, have been removed from this organ.

The embryonic form circulating in the blood vessels must evidently be removed from the body of the primary host by some species of blood-sucking insect. Manson thought that by virtue of the appearance of these embryos in the peripheral circulation during the daytime some day-biting insect must be responsible for the transfer. He suggested the mangrove fly, *Chrysops dimidiatus* v. d. Wulp, a common form in the region in question. These conclusions were attacked by Annett, Dunton, and Elliott (1901) without their being in position to furnish any very decisive evidence for the view they advance of the identity of *F. diurna* and *F. nocturna*. More recently Brumpt has brought forward strong evidence in favor of Manson's view in that he has discovered embryos of *F. diurna* in the circulating blood and identical forms in an adult female *F. loa* from the same host. He noted also that the embryos were constantly present in the peripheral circulation, even though more abundant by day than by night. The effort to discover the intermediate host in a species of *Glossina* was unsuccessful. If the observation of Brumpt that embryos are constantly present in the peripheral circulation is confirmed, then it is evident that the intermediate host may be a mosquito as in the case of other species of *Filaria*. Annett, Dutton, and Elliott found that *Anopheles costalis* served in West Africa as intermediate host for *F. bancrofti* but not for *F. diurna*; but this observation will not exclude other mosquitos also. However, it does speak strongly against their view of the identity of these two microfilariae.

Whatever may be the precise character of the intermediate host, of the changes passed through by the embryo filaria within it, and of the method by which it is introduced into the human body again, it is evident that the actively migrating *F. loa*, that form best known from cases on record, is the semi-adult worm. In some cases this has appeared within about one year after the host

has entered infected territory, and in other cases as much as 5, 11, or even 13 years have elapsed since leaving such infected regions before the parasite has made its final appearance in the eye. During this time it has undoubtedly made some growth, and at the end of the wandering stage it tends to settle down in deeper tissue. Here the female probably gives birth to the characteristic multitude of embryos which in the circulating blood await the chance of being drawn out into a suitable intermediate host to follow out again the same life-cycle. The adult ultimately becomes encysted and calcified by the activity of the tissue of the host, and Brumpt found four out of five adults in this condition in the case he observed.

It will be noted that in reality the discovery of *F. loa* in the eye of a patient in whose blood *F. diurna* is present, cannot be more than an indication of the relationship of the two; for if the view just advanced is correct, the wandering form is not fully mature, and consequently the embryos, if present, must come from *F. loa* of an earlier infection, and not from the form observed at the same time. This would evidently serve to explain the absence of embryos in those cases, such as Robertson's, already noted, where male and female were taken from the eye, and yet blood smears from the host showed no microfilariae present. Among natives in a badly infected region successive infections will be the rule, and wandering semi-adult forms will coexist along with the parturient females in deeper tissues and embryos in the circulating blood. In hosts infected during a briefer residence in the infected region, such conditions would be little likely to obtain, and embryos would be sought successfully in the blood only after the cessation of these migrations, when the worm is said by many to have disappeared from the body.

What time interval is necessary for the attainment of the full-grown form is not clear. Certainly migrations continue for many years after infection. In the extreme cases noted a worm was removed from the eye 13 years after the patient had left infected territory (Case 35); and in another, also recorded by Robertson, the parasite is said to have shown itself at irregular intervals for 15 years before final disappearance into deeper tissues. Among na-

tives frequent cases of infection in early life have been noted; thus the few cases first recorded from the West Indies include two of children, while in Europe that of Lacompte (No. 33) concerns an infant negress. In Africa one missionary notes that the work of the native children in school is interrupted by the periodical visits of the parasite to the eye. This early infection in the case of natives will insure the attainment of maturity by the parasite and the presence of embryos in the blood of the adult negroes, even though the development of the parasite proceeds very slowly, while the same slowness in development would render it unlikely that embryos could be obtained from the blood of hosts who had been exposed to infection first in middle life. This would serve to explain the absence of embryos from individuals as heavily infected as Robertson's case, which, even 10 years after the first infection, had no embryos in her blood (cf. the recent account of this case in Habershon,* 1904).

Looss (1905:167) has already called attention to certain differences in appearance between the illustrations of *F. diurna* given by different authors. This indicates either a confusion of what are distinct species, as he suggests, or slight differences in structure, due to age of the embryos and accompanying growth or ecdysis. The descriptions of these microfilariae are so general as to render a precise comparison difficult. In fact Brumpt originally regarded the embryos which he observed in the circulating blood as a new species which he denominated *F. Bourgii*, but later acknowledged their identity with *F. diurna*. It remains uncertain even yet whether the latter name may not include more than a single species.

TAXONOMY.

All the earlier observers regarded the eyeworm as an erratic Guinea worm. In 1805 Guyot recorded the evidence, already quoted in this paper, which led him to the view that it was distinct from that species. He also noted the name *loa* under which the form was known to the natives. Later authorities denominate this a generic term for worm rather than a distinct designation for this form.

*Ziemann (1905: 421) emphasizes the difficulty of determining the fact, and says that to demonstrate the embryos in the blood it is necessary often to try for several days and nights and to take blood from the region of the swellings. The distribution of the microfilariae in the body is exceedingly irregular.

Despite Guyot's view of its specific distinctness, the parasite continued to be confused with other forms or to be denied specific rank as late as 1851, the appearance of Diesing's monograph. In 1881, after having had opportunity to examine a specimen sent from Loango, Leuckart passed definitely and favorably upon the question of its distinctness; and in 1886 Blanchard's paper settled finally the rank of the species. The work of many later authors has aided in strengthening the position then assigned to it.

The parasite certainly belongs to the genus *Filaria* as now generally accepted; and the proposal of Diesing and Cobbold to transfer it from this to the related genus *Dracunculus*, which includes the Guinea worm, was so evidently an error that in a later edition Cobbold himself reversed his former action.

The synonymy of the species is confused, and depends in part on the positive determination of specimens for which no accurate data can ever be given. A partial list of the names used by various authors is given here for reference. This much may be said concerning the list: Guyot, generally quoted as 1778, but apparently first published in 1805, does not use the term *Filaria loa* to designate this parasite, nor apparently any other binomial form even though he does employ the common name of *loa*. If this be correct, the term can not rest back upon this author and date. Cobbold (1864: 388) seems to have been the first to use *loa* distinctly as a specific name. No one of the other terms listed below could be employed since the terms *F. lachrymalis* and *F. oculi* are preoccupied and *F. subconjunctivalis* cannot be assigned to its supposed authority, but is of much later date. Probably, then, *F. loa* will stand.

Filaria medinensis Gmelin 1788, in part.

of Diesing 1851, in part.

Filaria lachrymalis Dubini 1850, nec Gurlt 1833,

Dujardin 1845: 46.

Filaria oculi humani Dujardin 1845: 46.

Filaria oculi Gervais et van Beneden 1850: 142; nec von Nordmann 1832.

Moquin-Tandon 1859, in part.

De Bonis 1876: 129.

Filaria subconjunctivalis Guyon 1864 of Braun 1902.

Guyon himself does not use this term anywhere in this paper or in any other that I have seen.

- Filaria loa* Guyot of Leuckart 1876: 619.
 of Davaine 1877: cvii and 839.
 of Cobbold 1879: 205.
 of Blanchard 1886.
 of Stossich 1897: 21.
Filaria loa Guyot 1778 of Railliet 1893.
 of Braun 1895.
 of Moniez 1896.
 of Braun 1902.
Dracunculus oculi Diesing 1860: 697.
 loa Cobbold 1864: 388, 89.

GEOGRAPHICAL DISTRIBUTION.

The first six cases of *Filaria loa* recorded were all from the West Indies and the adjacent coast of South America, while among the first 21 cases listed 12 were from that same region and only nine from Africa. In all of the cases from the West Indies and South America the hosts were negroes with the exception of the creole child of Case 6.

As already noted, this case would seem to indicate the existence at that time (approximately 1795) in St. Domingo of an endemic center for this parasite. But this is the only evidence that *Filaria loa* has at any time gained a footing in the lands into which it has been introduced. It is noteworthy that since 1845, the date of Case 21 mentioned above, no one has recorded the occurrence of this parasite in the West Indies or in South America. Apparently its occurrence in that region stopped with the cessation of the slave trade, for all of the cases noted were in negroes; and in some cases it stands definitely recorded that they had come from Africa and three at least were from the coast of Guinea. Thus the worm which Mitchell saw in 1845 (Case 20) had apparently been seen eight years before, and the host, a young negress, had come from Africa in 1834. The single exception, beyond Case 6 already discussed, was in Case 19 where the worm was removed from the orbit of a negress said to belong to the Mina race of Brazil. It must be noted that at best the determination of the species in these 20 cases is only probable and confusion with *Dracunculus medinensis* is not excluded, while possibly rare cases of native American species showing similar habits may also be included. In any event it is important to note the complete disappearance of these cases from

the West Indies and South America just about three-quarters of a century after the first one was recorded. Thus far also the negro race might be looked upon as the distinctive host of this parasite, as indeed some authors maintained even much later than this date.

The first recorded specimens which had been taken from Caucasians were described by Morton (1877) and Bachelor (1880), while the next one, that sent Leuckart from Loango and described by him in 1881, is also the first one positively identified as a distinct species capable of differentiation from the Guinea worm, with which the majority of previous observers had classed this parasite. Following close upon this case numerous others in Caucasians definitely established the fact that the parasite exhibits no racial preference in its hosts.

The first case recorded in Europe was that of the French physician Lota (Case 30), who had previously lived in Gaboon, and after his return to France found himself infected. In this case the parasite was not removed. In France there have been listed four other later cases (Nos. 51, 52, 74, and 78), in all of which the parasites were removed. All five cases probably originated in the French Congo. A time interval of 15 years separated the first from the other three.

The French Congo was also the probable source of infection in the single case in which the worm was extracted in Switzerland (No. 31) and in that from Belgium (No. 33). The first specimen extracted in Germany (No. 42) probably came from West Africa, even though the extensive travels of its host render the exact region of infection impossible to determine; the second (Case 81) was brought from Kamerun. In England six specimens have been removed. In the first five cases the source of the infection was Old Calabar, in the last it was the Congo. Australia has had one case (No. 46) in a host who had resided previously on the Gold Coast.

Regarding the presence of *Filaria loa* in the western hemisphere, Clemow (1903:610) writes that "formerly it was said to have been seen from time to time among negroes in America, but since the slave trade from Africa to the New World has ceased this parasite is no longer found on the other side of the Atlantic." This

statement holds good for the West Indies and South America, where, as already noted, no cases have been recorded since 1845. But as regards the northern hemisphere, it is doubly incorrect, both as to former times and as to present records. On the one hand, it is noteworthy that no cases are listed on the North American continent from the days of the slave trade. One can hardly believe that such did not occur, but they seem to have escaped record in the literature so far as I have been able to follow it. On the other hand, there are not wanting recent cases in North America. The first case which actually occurred within the United States (No. 31) was reported in 1890. Here the host had sheltered four of these parasites, three of which were removed in this country. There are to be sure earlier records of *Filaria loa* in American literature, for Leidy had examined and reported briefly in 1877 'on a specimen sent Morton from Gaboon by Rev. Dr. Nassau, an American missionary. Also in 1880 Dr. Bachelor reported on a specimen he sent from Gaboon, said to be the first perfect specimen of *Filaria loa* seen in the United States.

The second specimen reported in this country was that of Milroy, which I recorded in 1902, and previous to the appearance of the present paper no others were found on record as having been removed in the United States. In the preceding pages (pp. 55, 56) I have discussed two specimens of Loveland, one of Friend, and one of Vail which must be added to the list. Of these six specimens the first was probably acquired in the French Congo and the other five in Kamerun where the hosts had been resident. Two cases (Nos. 68, 69) have been recorded from Canada in 1903, and are fully discussed in the preceding pages. It is noteworthy that all of the persons affected were missionaries in those regions, and all but one had suffered from the presence of more than a single specimen of the parasite, which fact points distinctly to its prevalence in the regions in which they had lived. In further support of this view may be cited also their own testimony on this point as already given.

In the foregoing paragraphs have been analyzed all cases of this parasite from other regions than Africa, and it has been shown that they are widely scattered both in time and in space, and also

that in all cases there is an apparent connection with a previous residence of the host on the African continent. It is accordingly fit to examine more in detail the evidence concerning the abundance and distribution of *F. loa* in that continent.

All records indicate that the west coast of Africa is the proper home of the parasite. One case, which is reported from Sierra Leone, marks its northern limit of extension. And even here the author (Prout, 1902) emphasizes the fact that no previous cases had been reported in this region, and that the patient had been living on the Congo, so that the infection probably occurred in the latter place. The specimens of Looss (1904) came from the Gold Coast, but no further information as to their source has been published, nor are other cases from this region on record, although the host in Case 46 is believed to have become infected in this territory and said such cases were common there.

From this point onward along the coast toward the south every territory has furnished many records of this disease. In Nigeria 10 cases are on record in my list, from Old Calabar 5 cases, from Kamerun 10 cases, from French Congo 21 cases, from Angola 6 cases. Eight cases are not precisely located, but belong to some part of this western coast. In addition it has already been noted that the 36 cases of this parasite from Australia, Europe and America owe their infection with great probability to this same region, 8 being traced clearly to the Congo, 8 to Kamerun, and 6 to Old Calabar, while in one case the host has visited this entire region at intervals.

Clemow is in error when he writes (1903: 610) that it seems to be absent from Kamerun. In a monograph on the Kamerun coast Plehn (1898) recorded four cases in man and other facts regarding this parasite which demonstrate unmistakably its endemicity in that region. To this evidence one must add that given in the present paper on cases in Americans who were undoubtedly infected in that same state where they resided as missionaries for some time.

These facts indicate that the parasite is distributed over the entire coast from about 5° north of the equator to at least 10° south, and various observers say that in certain regions nearly every inhabitant suffers from it. This is recorded for the Ogové River

by Miss Mary Kingsley, the well-known African traveler (1897:686), and for Kamerun by Lippert (Vail, 1905).

How far it may penetrate into the interior of the continent is as yet unknown. Certain it is, however, that cases occur more than 120 miles from the coast (Yarr, 1899), while a recent paper (Brumpt, 1904) records its presence in a postmortem made in Kassai, approximately 600 miles from the coast on one of the chief tributaries of the Congo. More precise knowledge of the life-history, especially of the intermediate host, and means of transfer of the species would enable one to give a better estimate of its range. Apparently the blood-inhabiting embryos which are now regarded as belonging to this species have a much wider distribution than *F. loa* itself.

Thus it is true that *Filaria diurna* has been recorded as far inland as Uganda, Central Africa, where Cook (1901) saw two cases. One should bear in mind that our knowledge of the microfilariæ is not sufficiently exact to enable the positive assertion that no other form exists in Africa which might be confused with the embryos of *Filaria loa*. But granting the certainty of the determination, there yet remains reasonable probability that the men in question were infected at a distance from the place in which they were examined. Cook also records in Uganda one case of *Dracunculus medinensis*, showing the tendency of movements over the great trade routes of the continent to bring together this species and *Filaria loa*, which in general are independent and, so far as present records show, do not occur together in any region.

The occurrence of *Filaria loa* in negro slaves, in travelers, in government officials, and in missionaries, points out distinctly the certainty with which any kind of intercourse between nations and geographic areas tends to transfer to new races and territories the diseases of the old. Increased means of communication and growing freedom of movement contribute clearly to the spread of maladies, and call for better means to check their advance into new regions. It is not to be doubted that some of the persons who brought *F. loa* into the United States now harbor its embryos in the blood. Though we know nothing precise of its life-history, the possibility lies close at hand that some blood-sucking insect may furnish

these embryos conditions for further development and may thus bring about the introduction of a new disease into our territory. Such cases as those of *F. loa* show clearly the gradual spread of disease through national intercourse.

4. PATHOLOGY OF *FILARIA LOA*.

SEAT OF THE PARASITE.

In many cases no more definite information is given than that the parasite occurred in the eye. In the absence of more specific details, this may probably be construed to mean crossing the eyeball beneath the conjunctiva, but above the cornea or sclerotic; in numerous cases, indeed, such a location is definitely assigned to the parasite. All in all, this is the most usual position of *F. loa* in the cases thus far on record. However, for reasons to be given later, it is probably only an accidental occurrence and not the normal seat of the parasite. While most frequently recorded on the surface of the eyeball, yet accurate records are not wanting to show that the parasite does occur, if infrequently, within the bulbous oculi. From the anterior chamber *F. loa* was removed in the case of Mercier (No. 4, but not in No. 5, as Kraemer incorrectly says), also in the case of Bachelor (No. 27), of Lacompte (No. 33), and possibly of Barkan, if this most doubtful account be interpreted as concerning *F. loa*.

From the lens this species has not been extracted, and those cases in which such a form has been reported from the vitreous humor are most uncertain. They rest in the main upon determination in life by the ophthalmoscope. But this method of procedure has resulted in some cases at least in confusion with a persistent hyaloid artery of peculiar form, as in the descriptions of Eversbusch, Fano, Malgat, Quadri, and Schöler, while the oft-cited account of Kuhnt concerns a peculiar small nematode, certainly not the species under consideration.

Roth is of the opinion that these parasites leave the eye by way of the nasal duct. More probably this is only apparently true, since, as Dr. Friend suggests (p. 39), the worm nearly always goes out of view by way of the inner canthus.

Outside of the eyeball *F. loa* has been reported at least 11

times as occurring in the eyelid, both upper and lower lid having been infected. From this position it has been removed six times or more.

F. loa has also been reported as wandering back into the orbit, as in Cases 14, 19, and 22; and while none of these cases is beyond doubt as to the species in question or the location of the parasite, there seems to be no question, on the other hand, that the loose connective tissues of this part afford the most ready resting-place from which the parasite may make its excursions over the cornea at short intervals, as reported by several observers.

When in other parts of the body than the eye, the parasite eludes observation in general, but it is important to note that nevertheless it has been seen and extracted many times in other regions, especially in the subdermal connective tissue. Thus it has been observed to cross the bridge of the nose from eye to eye (Case 38); it has been excised from below the loose skin of the back (Cases 31, 54, 88); from above the sterno-cleido-mastoid muscle (Case 94); from above the sternum (Case 69), and the left breast (Cases 72, 73, 94); from the lingual frenum (Case 74); from the loose skin of the penis (Case 59); it has been seen beneath the skin of the fingers, both in himself and in others, by Rev. Dr. Nassau, a missionary long resident in Gaboon, and well known as a student of the religious and social customs of the negro races; it has also been extracted from the metacarpo-phalangeal articulation (Case 87). Ziemann (1905) records that the worm is said by his patients to wander about under the scalp, and others maintain its presence in various other parts of the body. According to report of postmortems, the adult form occurs almost anywhere under the skin, but especially in the appendages (Cases 78, 80).

In view of all the evidence, the superficial connective tissues must be regarded as the true seat of the adult parasite, and its occurrence in the eye, or indeed in other adjacent parts, is more or less accidental and occasional.

EFFECT ON THE HOST.

When in the eye, *F. loa* is the cause of temporary piercing or lancinating pains as it makes its way through the connective tissue. This pain is also accompanied by the sensation of a foreign body

in the eye, and in case it crosses the field of vision there is added an uncertain image of the object. Both the pain and the sensation of the presence of some foreign body cease promptly with the withdrawal of the parasite into deeper tissues, while even repeated visits leave no permanent effect upon the organ other than to produce a very slight elevation of the conjunctiva, as Lota reports from observations on himself (Terrin, 1884), which one may consult (p. 76) for further details. In fact, the annoyance is so slight and of such brief duration as hardly to call for medical aid at all. Removal from the eye is not difficult when regard is had to the activity of the parasite and its tendency to flee at once when touched by any instrument. Even the natives in Africa practice its extraction with the rudest sort of instruments, in some cases using only a hooked thorn. In the earliest cases observed by European physicians it is recorded that such removal is unaccompanied by any untoward symptoms and is followed by complete recovery in a very brief time. So far as I have found, the same results uniformly follow the removal of the worm from the anterior chamber as well as from below the conjunctiva.

In the eyelid the *loa* is apt to give rise to a slight tumefaction at least, and this may stimulate entirely different conditions. Thus in the case recorded by Dr. Thompstone (No. 36) the parasite lay in the lower lid at the inner canthus close to the lachrymal sac, the swelling in that region giving the appearance of dachryocystitis. When an effort was made to press out the contents of the sac, the worm wriggled away.

F. loa may migrate from point to point under the skin without producing any visible effect upon the parts invaded. Thus in different cases it has been watched in its migrations from the eye to the forehead, or over the bridge of the nose to the other eye, or under the skin of the back or chest; and in all of these it is not recorded that any modification of the normal appearance of the part followed the movements of the worm. One of the most distinct and trustworthy of these observations is that quoted from Starr in the present paper (p. 40).

CALABAR SWELLINGS.

The first publication I have found on the nature of Calabar swellings (the *Kamerungeschwülste* of the German authors) are in the book by Plehn (1898), and a contribution exclusively on this topic by Thompstone (1899), a district medical officer in Old Calabar. To be sure, they were recognized as a distinct disease much earlier, and are referred to under this name by Robertson (1895). Since then numerous references have been made to their occurrence and several observers have discussed at length their character and cause. They are apparently spontaneous and fugitive in character, appearing suddenly and requiring two to three days to disappear. In size half that of a goose egg, they may occur on any portion of the body, though, according to most, they apparently favor the extremities. They are painless and do not pit under pressure. According to Thompstone, they come one at a time and recur at irregular intervals of time. He also states they are somewhat hot both objectively and subjectively, while Joseph (1903) states distinctly that they are accompanied by no temperature.

Robertson (1895) was apparently the first to call attention to the fact that his patient afflicted with *F. loa* also suffered from Calabar swellings. Later observations on the same patient record (Robertson, 1897) an immediate recurrence of the trouble on return to Old Calabar, where itching behind the eyes and swellings on the arms are almost universal among the natives. He also says that when the parasites are felt moving, headache and nausea as well as puffy swellings of the arms are troublesome, while all parts of the body may be affected, especially the scalp.

In regard to the cause of these swellings, Manson (1903) sums up the case well when he says: "Their peculiar geographic range, which, it would seem, includes the Congo basin, the fact that they come and go, the fact that they persist in recurring after the subject has left the endemic districts, render it practically certain that they are of parasitic origin." In the same paper he reports a series of eight cases of the disease among missionaries on the Upper Congo, two of which had been under his personal care. He further notes the general association with *F. loa*, and conjectures that they may be due to the parturition of this species. Their association with *F.*

loa and possible relation to that parasite had already been commented on by Robertson. The absence of *F. diurna*, the conjectured embryonic form of *F. loa*, as shown apparently by his blood tests, may easily be due to failure to make preparations at the proper time or place. Furthermore, the geographic distribution of this malady is much the same as that of *F. loa*, which would further strengthen the view that there exists a causal relation between the two.

More recent publications have brought forward additional proof of this causal relation. Thus Habershon (1904) has presented strong evidence in favor of the view when he reports that almost every European at Yakusu suffers, and adds details of several cases which were under careful continuous observation and showed the presence also of *F. loa*. In one case the attack commenced with the most intense neuralgic pain, followed by swelling of the part affected, which began a few hours later and was comparable to an attack of acute myositis. Kerr (1904) also adds evidence on the relation between *F. loa* and the Calabar swellings in a series of four cases. Apparently Ziemann (1905) has been able to demonstrate the embryos in the swellings, as he explains the difficulties attendant upon the demonstration. He seems to think, however, that these swellings are due to *F. perstans*, which in his opinion is the embryonic *F. loa*, while *F. diurna* does not differ from *F. bancrofti*. He is in accord with previous authors in holding that *F. loa* remains mostly hidden in its wanderings, but causes inflammation in the subdermal connective tissue. Wurtz et Clerc (1905) found in their case of infection with *F. loa* that a tumefaction was produced on the right cheek when the parasite was wandering about in the region, of the eye. They also added the important observation that a pronounced intense eosinophilia was associated with the presence of *F. loa* in the system. It should be noted that the general symptoms of this case point unmistakably to the presence of a number of parasites, and the extreme character of the eosinophilia noted was perhaps due to the multiple infection.

The view that in some way Calabar swellings are related etiologically to the parasitism of *F. loa* rests thus on strong presumptive evidence, and it is timely to consider the theories which have

been offered to explain the pathological conditions noted. It is clear, without further discussion that the mere presence of the parasite, as of a foreign body of equal size, would not be sufficient to evoke the swellings. It is equally evident that the constant limitation of the worm to the connective tissue, especially in the subdermal region, would throw out of consideration the introduction, even occasionally, of foreign matter of any sort, and limit the problem clearly to the parasite itself and its own activities and products, working upon the normal tissues with which it comes in contact.

The earliest suggestion made was that of Robertson that to the migrations of the *loa* are due these swellings which are associated with its presence. Careful study of the data recorded in connection with the various cases seems to show, however, that mere movement cannot be the exciting cause. Note first that the swellings are local and infrequent. Now, mere migrations, if effective, ought to produce linear tumefactions conforming to the path the worm has followed, if not immediately coincident in time with its movements. There is one record of such movement of the swelling, given by Milroy in the present paper (p. 75), but another similar observation has not been noted, and there is much indirect evidence to show that it does not occur ordinarily at least. In fact, these swellings are usually described as oval, circumscribed, and of relatively small size; hence the stimulating factor must be a variable or occasional one. Furthermore, the swellings are single or rare, while it is indisputable that the migrations of the worm are constant and considerable. Its activity and freedom of movement have been commented upon by many observers, and are manifest both in the eye and elsewhere in the body. This striking contrast between the pathological conditions and the parasite supposed on good grounds to produce them can be explained only by the assumption that the exciting factor is an intermittent element in the biology of the parasite, of relatively infrequent occurrence. In further support of the view that the pathological condition is not the result of the mere movements of the parasite may be urged the record of observations concerning its movements under the conjunctiva. The unanimous testimony of observers is that the parasite produces no change whatever in the appearance of the organ,

as it moves across the surface of the eyeball, nor is any alteration visible subsequently save an insignificant elevation of the surface. Similarly it has been seen moving under the skin in other parts of the body without pathological changes resulting. Important additions to the previous records on this point are found in the observations of Friend, Starr, and Loveland included in the preceding pages.

Manson reports the statement of one of his patients that the erythema and swelling are due to mechanical excitation when the region of the parasite is rubbed. Indeed, this lady, long resident in Old Calabar, informed him that if rubbing or scratching is not indulged in when a *loa* approaches the surface, there will be no swelling. It is difficult to see how the rubbing could produce such definite areas, or how the number and frequency of the swellings could be so limited. Furthermore, Milroy records the evidence of his patient that such a swelling may be rubbed to remove it, so that the evidence is at least somewhat confused.

It can hardly be that the parasite in its migrations stimulates nerve fibers or endings, and thus produces as secondary results the conditions; for any excitation of sensory elements is inadmissible as the swellings are distinctly declared by most authors to be painless. Hence Blanchard's suggestion of similarity to the symptoms evoked by *Hypoderma lineata*, a fly larva that carries out subcutaneous migrations, hardly meets the conditions of the case. Looss (1905) calls attention to a more striking parallel between the Calabar swellings and those seen at times in cases of *Sparganum mansoni*, a migrating cestode larva which occurs in the East.

Hardly more acceptable as an explanation of Calabar swellings is the view that the *loa* in its movements stimulates unduly reflex or sympathetic fibers. The infrequency of the swellings, and their distribution as compared with the nervous elements, speak distinctly against the supposed relation.

Convinced of the insufficiency of previous efforts to explain the tumefactions, Manson (1903) advanced the view that they are caused by the discharge of embryos into the tissues. This discharge of embryos from the parental form is intermittent, and would produce the swellings by acting as a mild irritant and causing a transient

edema. In most respects this view meets the conditions thoroughly and it can hardly be said that definite facts are recorded as yet which it fails to explain. Yet its acceptance involves distinctly the concession that not all cases of infection with *F. loa* are subject to Calabar swellings; for when the host harbors the male parasite alone, or also only immature females, there can be no discharge of embryos into the tissues, and consequently no swellings produced. The theory of Manson conforms to the facts in so far that cases of *F. loa* are on record, and are also distinctly noted by physicians (Ziemann, 1905) in the infected region in which Calabar swellings do not occur. On the other hand, there are cases in which the swellings are found at such an early period after the coming of the host into infected territory that the *loa* could not have reached sexual maturity. As already emphasized, all the evidence points to an extremely slow growth of the parasite and to comformable delay in reaching sexual maturity. Unless this evidence has been entirely misconstrued, and a more rapid attainment of sexual maturity is possible under some undetermined circumstances, the view of Manson fails to account completely for the facts in the case. In the case of Milroy the swellings began within two years from the time of entrance into the infected region; furthermore, the parasite extracted three years later was a male, and, so far as known, only a single parasite has been present in the body of this patient.

In view of these facts, I venture to suggest another feature which may not be without its bearing on the production of these swellings. The parasite will from time to time discharge from its body waste materials which in their very nature are toxic, and hence likely to cause such local changes as the Calabar swellings. The action in this case would be chemical rather than the mechanical irritation from the discharge of embryos. The ultimate decision in this matter must necessarily await the accumulation of further evidence. So far as facts at present on record are concerned, none of the causes advanced thus far is sufficient to explain the rarity of the tumefactions in cases of multiple infection by the parasite.

5. CLINICAL DATA.

CLINICAL NOTES ON CASES NOS. 53, 54.

By W. F. MILROY, M. D., OMAHA.

Batanga is a settlement situated in German territory, on the western coast of Africa, in about 3° north latitude. In 1897 Mr. K., an American of German parentage, became a resident of this place. About two years later he first observed upon his body a tumefaction which, in that country, is known to foreigners as "the African swelling," and which is by common consent attributed to a parasite. To the natives this parasite is known as the eyeworm because of a disposition it exhibits to frequent the neighborhood of that organ.

That the swelling is caused by this parasite seems not to be a fact absolutely established. However, upon one occasion within the knowledge of Mr. K., one of these upon the dorsal surface of a woman's hand, being rubbed with a view to removing it, the parasite was seen to emerge from the tumefaction and make its way across the metacarpophalangeal articulation, from which location it was extracted. Upon another occasion the swelling appeared, upon his own person, over the right frontal eminence. Within an hour it had extended downward across the supra-orbital arch, along the right side of the nose, and outward beneath the eye as far as the outer limit of the orbit. During this process, when the swelling reached the side of the nose, the movements of the parasite became visible beneath the skin and upon the surface of the tumefaction, where it was seen to cross below the eye, and the sharp, stinging sensation was apparent to its host as it made its way downward across his cheek. From these and similar observations there would appear to be little room to question the correctness of the assumption that the occurrence of this swelling is conclusive evidence of the presence of the parasite.

The swelling is from three to five or eight centimeters in diameter and not greatly elevated. The appearance of its cutaneous covering remains normal. A sharp stinging or smarting sensation, with more or less itching, attracts attention where the swelling is about to appear. The swelling is indurated and is sometimes accompanied by great pain, but in other cases pain is absent. From its first appearance until the part returns to its normal condition a period of two or three days elapses. It occurs upon the head or face, the wrists, hands or fingers, the ankles, feet or toes. It is seldom seen upon other parts of the body. This may be accounted for by the relative deficiency of subcutaneous connective tissue in the parts mentioned. When the joints of the extremities are involved, marked stiffness and pain are felt on motion, and in some instances creaking of the joint is so marked as to be audible at a distance. No distinction of age, sex, or condition exists as to susceptibility to invasion of this parasite. The host is never aware of its presence except when it approaches the surface of the body, and no constitutional symptom is recognized as due to its presence. So far as known to Mr. K., no permanent harm has ever resulted from its occupation of the human body.

In February, 1902, Mr. K. came to me for the removal of his "eye worm," which had made its appearance beneath the cutaneous surface of the upper lid of his right eye. Its movements were readily visible. A fold of the skin was firmly seized with a forceps so as to include the moving body, an incision was made near the forceps, and after a search of 15 or 20 minutes a portion of the parasite appeared in the wound; this was seized and the animal extracted. Since the first appearance of the parasite in his body, in 1899, it had shown itself at points as remote as the sole of the foot and the face.

He sometimes was unaware of its presence for two or three months continuously, but it was usually manifest at much shorter intervals. Previously unsuccessful efforts had been made to remove it from the inner side of his left arm, below the nipple on the left side, and near the lower angle of the left scapula. As a rule, having appeared at an accessible point, it was gone before a surgeon could be reached. Mr. K. stated that he had experienced no inconvenience from the presence of his tenant, except when it approached the surface of his body, where it usually remained but a very short time. On one occasion it gave rise to violent pain as it made its way across the sclerotica below the iris of the right eye.

Since the extraction of this specimen four months have elapsed with no recurrence of the symptoms. Whether more than a single specimen has ever existed in the body of this gentleman he does not know, but during the period of about three years, from the first sign of its presence until the extraction of the parasite in February last, the characteristic manifestations have never appeared simultaneously at more than one point.

OBSERVATIONS OF TERRIN.

To these notes of the case of Milroy it is valuable to append another little known record quoted by Terrin. It is an interesting account, which gives the following clinical picture of *F. loa*, and comes from the observation of a French physician Lota who had opportunity to study the action of the worm in his own eye.

After his stay in Gaboon and return to France, Lota suffered oft-recurring conjunctivitis, with which he was previously not afflicted. Without warning he felt in the right eye a sting without outward cause, and a feeling of heaviness which was unpleasant, while at the same time there arose an active injection of the conjunctiva bulbi. These symptoms disappeared on application of cold lotions to the eye, but recurred in a few days. Lota attached no importance to the matter. Five months after his return he was awakened from sleep one morning by a sharp pain in the right eye. He had the sensation of a foreign body under the upper lid, accompanied by frequent winking. As he drew up the upper lid before a mirror, he noticed the conjunctiva was reddened, swollen, and slightly elevated. He recognized under it a yellow irregular mass, without being able to determine its nature. The sensation of a foreign body lasted about two hours, and then disappeared suddenly. Lota investigated the eye again and could determine only a slight conjunctivitis; the yellow body was gone!

That evening the same symptoms came on again. Lota noted on the sclera a yellow, round body of the caliber of a knitting-needle, about 2 to 3 cm. long, which moved itself from the external angle of the eye toward the caruncle, at times straight, again bending itself into **U** and **S** shapes; it crept along under the corium above the sclera, only to disappear at the inner angle of the eye. Next evening the worm showed itself under similar circumstances below the conjunctiva above the cornea; here it remained a long time, so that several colleagues of Lota could observe its presence and movements. It then disappeared again into the depth of the eye and never appeared thereafter. Its presence had induced no further change on the bulb than an insignificant elevation of the connective tissue. The visual power was never disturbed.

6. ANNOTATED BIBLIOGRAPHY OF *FILARIA LOA*.

The accompanying bibliography includes all references to *Filaria loa*, and also all which at any time, so far as I have been able to ascertain, have been construed as such, together with the publications which have contributed to the explanation of any doubtful cases. No attempt has been made to include all references to human eyeworms other than *F. loa*, although the involved condition of this subject has resulted in bringing together here the majority of these also. The list includes only the more prominent textbooks, or such as contain extended discussion or original contributions to a knowledge of this species. For valuable assistance and criticism in the preparation of this bibliography I am indebted to Professor J. I. Wyer, librarian of the University of Nebraska.

In printing these references the two numbers separated by a colon denote volume and page; i. e., 28: 510 means Volume 28, page 510. An additional number in parentheses before the two just explained denotes the series, and is used only when the volumes of each series are numbered separately from those of preceding series.

ADDARIO, C. 1885. Su di un nematode dell'occhio umano. *Ann. d. ottalm.*, 14: 135-48. 1 Pl.

This much-cited paper deals with another species, *F. conjunctivae*, identical with the form described later by Grassi as *F. inermis*, a normal parasite of horse and ass, and an occasional one in man. It is entirely distinct from *F. loa*.

ANNETT, H. E., DUTTON, J. E., AND ELLIOTT, J. H. 1901. Report of the Liverpool Expedition to Nigeria. Part II, Filariasis. *Thompson Yates Laboratory Reports*, 4: 1-93, 14 Pls.

Found *F. diurna* in a boy of lower Nigeria who was also infected with *F. loa*.

ARRACHART, J. N. 1805. Mémoires, dissertations et observations de chirurgie. Paris, 8vo, 302 pp. Mémoire sur les vers des yeux. Lu à l'Académie de Chirurgie en 1778. (p. 217).

Records the cases in St. Dominique known to him, and asserts that it is a valid species distinct from the Guinea worm. Reproduces Bajon, 1777, and Guyot, 1805.

ASSENOVA, SALA. 1899. Étude sur la provenance des entozoaires superficiels. Thèse. Fac. méd. univ. Nancy. No. 6.

Cites verbatim the case of Lota as Obs. 22, briefly that of Robertson as Obs. 23, and notes in text a few other cases without detail.

BACHELOR, H. M. 1880. The Eye Parasite, *Dracunculus loa*. (Letter to the editor from Gaboon, west coast of Africa, Dec. 15, 1879.) *Medical Record* (N. Y.), 17: 244.

Specimen extracted from native young man. The first perfect specimen sent to U. S. A.

BACHELOR, H. M. 1881. *Filaria loa* and *Pulex penetrans*. *Medical Record* (N. Y.), 19: 470-471.

Case in white woman, missionary: three worms removed. Dr. Nassau (cf. Morton, 1877) "had one in the areolar tissue between the thumb and index finger."

- BACHELOR, H.M. 1881a. *Filaria loa* and *Pulex penetrans*. (Trans. IV), *Bull. N. Y. Path. Socy.* (2), 1: 108-111.
Identical reprint of Bachelor. H. M., 1881.
- BAJON, M. 1777. Mémoire pour servir à l'histoire de Cayenne et de la Guyane française. Paris, 2 vols., 8vo. Abstr. in *Jour. de méd.* (1778), 49: 386-408, 481-97.
Two cases (Vol. I, p. 325) in negroes at Cayenne, in 1768 and 1771. Quoted verbatim by Blanchard (1890) under date of 1778. Also in Arrachart, 1805: 217.
- BAJON, M. 1781. Abhandlungen von den Krankheiten auf der Insel Cayenne und der französischen Guyana. Erfurt. Bd. I, II.
Not found. Cited after Kraemer (1899). Probably translation of Bajon, 1777.
- BARKAN, A. 1876. A Case of *Filaria medinensis* in the Anterior Chamber. *Arch. Ophth. and Otol.* (N. Y.), 5: 151-52.
Filamentous object adherent to iris was removed from native Australian and found on microscopic examination to be "*F. medinensis*." Can hardly be *F. loa*, but the absence of data leaves it permanently uncertain. Perhaps an Australian filaria.
- BARKAN, A. 1876a. Ein Fall von *Filaria* in der vorderen Augenkammer. *Archiv f. Augenheilkunde*, 2: 381-82.
Literal translation of Barkan, 1876.
- BARRETT, J. W. 1896. A Case of *Filaria oculi humani*. *Arch. of Ophth.* (N. Y.), 25: 291-92.
Worm removed in 1893 from eye of white male who had lived on Gold Coast and had come to Melbourne four years before. Identification probable. First case in Australia.
- BARRETT, J. W. 1897. Ein Fall von *Filaria* im menschlichen Auge. *Arch. f. Augenheilk.*, 34: 255. Cf. *CB. Bakt. u. Par.*, 22: 419.
Literal translation of Barrett, 1896.
- BERNARD, P. 1898. Un cas de *Filaria loa* mâle. *Arch. d'ophtalm.* (Paris), 18: 604-6. Abs. in *Jour. Trop. Med.*, 1: 110-11.
Removed from white male who had lived in Congo (1894-96). First seen about three years before removal. Identified by Blanchard; second case, first male *F. loa* seen in France.
- BLANCHARD, R. 1885-88. *Traité de zoologie médicale*. Paris, 2 vols., 8vo.
Exact account with full references to cases of *F. loa* (2: 10-12).
- BLANCHARD, R. 1886. La filaire sous-conjonctivale (*Filaria loa* Guyot). *Progrès méd.* (Paris), (2) 4: 591-93, 611-12. Also in *Rev. clin. d'oculist.*, No. VII, p. 159 (after Kraemer, 1899: 85).
Fine record of earlier cases with discussion of structure, life-history, and relation to eyeworms of other animals. Presence in American hemisphere attributed to slave trade.
- BLANCHARD, R. 1899. Nouveau cas de *Filaria loa*. *Arch. Parasitol.* (Paris), 2: 504-34. 12 figs. *Rev. CB. Bakt. u. Par.*, 28: 457; *Zool. Centr.*, 7: 243-44.
Complete discussion of twenty-five old and one new case, with full illustrations of structure and data on life-history and distribution. Good bibliography.
- BRAUN, M. 1902. Die thierischen Parasiten des Menschen. Dritte Aufl. Würzburg; 8vo, 360 pp., 272 figs. [Title-page date, 1903; received here in December, 1902.]
F. loa (p. 271); brief, accurate.
- BRUMPT, E. 1904. La *Filaria loa*, Guyot, est la forme adulte de la Microfilarie désignée sous le nom de *Filaria diurna* Manson. *CR. Soc. Biol.* (Paris), 56: 630-32.
In an autopsy on the Congo, *F. diurna* was found in the blood, also a fragment of an adult female filaria in the heart. The latter agreed in all details with *F. loa*, but the absence of head and tail made a final determination impossible. The author regards *F. loa* as an accidental parasite of the eye.

CHARLES, R. H. 1892. Case of *Filaria loa* by Dr. Argyll Robertson. *Sci. Mem. Med. Officers, Army India*, VII, No. 5.

Cited after Penel, 1905a: 157. Evident error as this is the precise location of a famous paper by this author on the Guinea worm which contains no reference to the *loa*.

CHIRALT, V. 1880. Sobre un caso di *Filaria oculi*. Att. session congress reg. de cien. medicas [1879], Cadiz. *Cron. oftal.*, Cadiz (1880-81), 10: 473-80.

Not seen; cited by Kraemer, 1899: 85. Probably identical with following reference.

CHIRALT, V. 1882. Sobre un caso *Filaria oculi*. Cadiz, Att. session congress reg cien. med. [of 1879], pp. 2-9, 1 fig.

Not seen; cited by Coppez (1894: 567) as *Filaria* in vitreous body, p. 473.

CLEMON, F. G. 1903. The Geography of Disease. *Camb. Geog. Series*; 624 pp., maps.

F. loa (p. 609) only on west coast of Africa, endemic from few degrees N. L. to about 10° S. L. Not in America since cessation of slave trade.

CLOT-BEY. 1832. Dragoneau. Revue générale Académie royale des sciences. Séance du 10 décembre. *Arch. gén. de méd.* (Paris), tome année, 30: 573.

Gervais et van Beneden (1859), Davaine (1877), and Rayer (1843) cite the reference as given. I have compared the original, and the citation is absolutely correct; there is no hint in the abstract of any other author. The brief description records an observation by the author of a worm in the orbit and crossing the cornea of a slave girl in Monpox, brought from Africa some years before. Yet, according to Guyon (1838), Clot-Bey says he never has been in America, and Leuckart (1881), together with later authors, declares the citation incorrect, and all attribute the case to Roulin, by what authority I have been unable to ascertain. It does not help the case to read in Guyot (1838): "Als ich mich im Jahr 1828 zu Monpox am Magdalenenflusse in Neugranada befand, führte mich ein dort ansässiger französischer Apotheker zu einer 25 bis 30 Jahr alten Negerin, die schon erwachsen aus Afrika herüber transportirt worden war," etc.

COBBOLD, T. S. 1864. Entozoa. An Introduction to the Study of Helminthology. London; 8vo, 480 pp., 21 pls., and 82 text figs.

Brief account under *Dracunculus loa* (p. 388). Defends its specific distinctness. Cites Davaine (1860) for further details.

COBBOLD, T. S. 1879. Parasites. London; 8vo, 508 pp., 85 text figs.

Transfers species back to genus *Filaria* (p. 205). Otherwise as before. Short bibliography.

COOK, A. R. 1901. Notes on the Diseases Met with in Uganda, Central Africa. *Jour. Trop. Med.*, 4: 175-78.

Records (p. 178) the occurrence of two cases of *Filaria diurna* in this region.

COPPEZ, H. 1894. Un cas de filaire dans la chambre antérieure d'un œil humain.

Arch. d'ophtal., 14: 557-62. Also in *Clinique* (Bruxelles), 1894, 8: 481-84. Rev. in *Arch. of Ophth.* (N. Y.), 1895, 24: 284.

In infant negress from Congo, worm in anterior chamber; immature.

COPPEZ, H. 1895. Progress of Ophthalmology. Review of Coppéz, 1894. *Arch. of Ophth.* (N. Y.), 24: 284.

CUNIER, FL. 1843. As editor reprinted Nordmann, 1843, and Rayer, 1843, with comments, etc. *Ann. d'oculist.* (Bruxelles), 9: 136-77.

DAVAINE, C. 1860. Traité des Entozoaires et des maladies vermineuses de l'homme et des animaux domestiques. Paris, 8vo, xix + xcii + 838 pp., 37 + 31 figs.

Full data on earlier cases.

DAVAINE. 1877. *Ibid.*, 2d. ed., Paris, cxxxii + 1003 pp., 72 + 38 text figs.

Apparently identical reprint (p. cvii + 839) of earlier editions as regards this species.

DE METS. 1896. Une observation de filaire de la rétine. *Belgique Méd.* (Gand-Haarlem), 1: 737-42. Abst. in *Ann. Ophth. and Otol.* (N. Y.), 5: 1097.

Nematodes in urine; not precisely described or identified. Not *F. loa*. Presence in retina inferred.

DIESING, C. M. 1851. *Systema Helminthum*. Bd. II. Vindobonae.

Under *F. medinensis* (p. 270): "Habitaculum, homo . . . rarissime sub conjunctiva oculi (Bajon, Mongin, et Blott)."

DIESING, K. M. 1861. Revision der Nematoden. *SB. math.-naturw. Kl., Akad. Wiss., Wien.*, 42, Nr. 28.

Lists *F. loa* as *Dracunculus oculi* (p. 697).

DRAKE, BROCKMAN. 1894. (Notes.) *Ophth. Review* (London), 13: 331. Also in *Ophth. Socy. Brit. Med. Jour.*, 1894, 2: 921.

In eye of young woman in Madras, India; worm escaped from puncture and not studied. Blanchard (1899) thinks this is more probably *F. equina*, common in India.

DRAKE, BROCKMAN. 1894a. Cas de *Filaria loa* sous-conjonctivale. *Ann. d'oculist.*, 112: 336.

Translation of Drake, 1894.

DUJARDIN, F. 1845. Histoire naturelle des helminthes ou vers intestinaux. Paris. 8vo, avec un atlas de 12 pls.

Notes (p. 46) the occurrence in the human eye of a *Filaria* not yet described, which is certainly different from *F. medinensis*.

DUYSE, VON. 1895. Un cas de Filaire dans la chambre antérieure d'un œil humain. *Arch. d'ophtal.* (Paris), 15: 701-6.

Same case as Coppez (1894), Gauthier (1895), and Lacompote (1894); worm extracted by latter. Discussion of species and records.

EVERSBUSCH, O. 1891. Entozoen im Glaskörper (Mittelfränkischer Aertztetag in Fürth). *Münch. med. Wchnschr.*, 38: 532. Also in *Jahresb. d. Leist. u. Fortschr. d. Ophth.*, 22: 305.

EVERSBUSCH, O. 1892. Glaskörperentozoen. *Ber. über d. XXI. Versammlung d. Ophth. Ges.* (Heidelberg, 1891), p. 249. Ausserordentliches Beilageheft zu *Klin. Monatsblätter f. Augenheilkunde*, 29.

Preliminary report on supposed living worm observed by eye mirror. Extended description not published as promised. Certainly not *F. loa*. Probably persistent hyaloid artery.

FANO, S. 1868. Observation de filaire vivante du corps vitré. *Union méd.* (Paris), 3 sér., 5: 389-391.

Observed by eye mirror in child of 12 years; not removed.

FANO, S. 1868a. Filaire vivante dans le corps vitré. *Ann. d'oculist.*, 59: 207-8.

Literal reprint of Fano, 1868.

FANO, S. 1876. Filaire vivante du corps vitré. Modifications survenues dans l'œil malade huit ans après le premier examen. *Jour. d'oculist. et du chir.* (Paris), 42: 172-74.

Examination of case of 1868 after eight years. Original text reproduced. Further observations unimportant. Not *F. loa*.

GAUTHIER, C. 1905. Microfilaires du sang coïncidant avec une filaire de l'œil. *CR. Soc. biol.* (Paris), 58: 632-34.

In a patient who had had a filaria in the eyelid were found later embryos like Manson's *F. diurna*, but smaller than Brumpt's measurements of the embryos of *F. loa*.

GAUTHIER, G. 1895. *Filaria oculi humani*. *Annal. de l'Institut chir.* (Bruxelles), Chap. Ophtalm., p. 15.

Not found; probably the same as the following entry.

GAUTHIER, G. 1895. Filaire de l'œil humain. (*Annal. de l'Institut chir.*, Bruxelles, 1895.) *Ann. d'oculist.*, 114: 152-53.

Only a short review by Dastot. Young Congo girl; worm moved rapidly through anterior chamber; not extracted; probably *F. loa*. According to Blanchard (1899), same case as Coppez (1894).

GERVAIS, P., ET VAN BENEDEN, P. J. 1859. Zoologie médicale. Paris: J. B. Baillière et Fils, 8vo, 2 vols., 198 text figs.

Case communicated by French marine surgeon, Lestrille, from Gaboon. 1854, given in full (p. 143). Older cases quoted.

GESCHIEDT, A. 1833. Die Entozoen des Auges, eine naturhistorische, ophthalmologische Skizze. *Zeit f. Ophthalm.* (Dresden), 3: 405-62.

An oft-cited reference which concerns a small nematode otherwise unknown (cf. Nordmann, 1832), and certainly not *F. loa*. Discussion of eye parasites in other animals.

GRASSI, B. 1887. *Filaria inermis* (mihi) ein Parasit des Menschen, des Pferdes, und des Esels. *CB. Bakt. u. Par.*, 1: 617-23.

Extended description of *F. conjunctivae* Addario 1885 (q. v.) and of the cases of this species sometimes confused with *F. loa*.

GUYON, [J. L. G.] 1838. Note sur des vers observés entre la sclérotique et la conjonctive, chez une négresse de Guinée habitant la Martinique. *CR. Acad. des sciences*, Paris, 7: 755-56. (Cf. Guyot, 1838.)

Case of Blott, who extracted two *F. loa* from a young negress of Martinique that had come from the African coast. The worms were sent to Guyon.

GUYON, [J. L. G.] 1841. Note sur un ver trouvé dans le tissu cellulaire sous-conjonctival. *Gaz. méd. de Paris*, 9: 106.

Corrects an erroneous reference to the preceding case as due to *cysticeri*, and quotes Blott as reporting the patient perfectly well to date.

GUYON, [J. L. G.] 1864. Sur un nouveau cas de filaire sous-conjonctival, ou *Filaria oculi* des auteurs observé au Gabon (côte occidentale d'Afrique). *CR. Acad. Sci.* (Paris), 59: 743-8.

One specimen taken from a negro of Gaboon, Africa, and extended reference to six previous cases, all from America. Gives as the first evidence of the occurrence of *F. loa* a plate printed in Frankfurt (Germany) in 1508. This much-cited illustration is shown by Ward (1905) to be fanciful.

GUYON, [J. L. G.] 1864a. Sur un nouveau cas de filaire sous-conjonctival ou *Filaria oculi* des auteurs, observé au Gabon (côte occidentale d'Afrique). *Ann. d'oculist.*, 52: 241-5.

Reprint of Guyon, 1864.

GUYOT. —. 1805. In Arrachart, 1805.

Copied by Rayer (1843) as Obs. IX. French naval surgeon records six cases in 1777 from African coast. Extraction attempted and failed.

GUYOT, —. 1838. Ueber Würmer welche sich unter der den vorderen Theil des menschlichen Auges bedeckenden, Schleimhaut aufhalten. *Froriep's Neue Notizen*, 8: 230-31.

Cites earlier cases; notes as new that of Blott who sent one specimen to Blainville. This paper is that referred to under Clot-Bey, 1832. This apparently should be Guyon, 1838, with which it agrees, though the German translator has printed consistently Guyot. This same error has been made more recently by Scheube, 1900.

HABERSHON, S. H. 1904. Calabar Swellings on the Upper Congo. [Includes letter from D. Argyll Robertson.] *Jour. Trop. Med.*, 7: 3-4.

Almost every European at Yakusu suffered. Clinical data. Several cases of *loa* briefly noted. Letter gives further history of Robertson's patient, including extraction of parasites not in eye.

HARRISON, J. H. H. 1904. *Filaria loa* (?). Selected Colon. M. Repts., 1901-2, London, p. 46.

Not seen. Cited from *Index Medicus*.

HENRY, F. P. 1896. Remarks on *Filaria*. *Proc. Acad. Nat. Sci.* (Phila.), 1896: 271-75. *Rev. Zool. Jahresb.*, 1896, Vermes, p. 44.

Cites cases and data from Manson and Robertson; rejects Manson's view that *F. loa* is adult of the embryonic bloodworm, known as *F. diurna*.

HIRSCHBERG, J. 1895. Ueber einen aus dem menschlichen Augapfel entfernten Fadenwurm. *Ber. klin. Wchnschr.*, 32: 956-58, 971. Rev. in *Zool. Centr.* 3: 233; *CB. Bakt. u. Par.*, 18: 755. Also 1896, *Verh. Berlin. med. Ges.*, 26, Pt. 2: 287-94; *Centralb. prakt. Augenheilk.*, 20: 27-32, 4 figs.

Female *F. loa* taken from eye of negro in Cayo, French Congo. Review of earlier cases.

HUBER, J. CH. 1898. Bibliographie der klinischen Helminthologie. Supplementheft Inhalt: *Filaria* (excl. *F. sanguinis hominis*), *Strongylus*, *Gnathostoma*, *Strongyloides*, *Rhabditis*, *Pentastomum*. Jena; 22 pp.

F. loa (pp. 3-5); doubtful cases (p. 6). References arranged by countries, brief annotations.

JOSEPH, E. 1903. Medizinische Mittheilungen aus unseren westafrikanischen Kolonien. *Deutsche med. Wchnschr.*, 29: 145.

Describes Kamerun swellings as occurring anywhere, but especially on extremities. Not painful; no temperature; cure spontaneous in a few days.

KERR, T. S. 1904. Calabar Swelling and its Relationship to *Filaria loa* and *diurna*. *Jour. Trop. Med.*, 7: 195-96.

Cites theories of Manson and Robertson regarding Calabar swelling. Records cases of Habershon, Wurtz, and Hanley. Thinks this evidence demonstrates relation of *F. loa* and its embryonic form *F. diurna* to the trouble.

KERR, T. S. 1905. [Abstract] *Arch. f. Schiff's u. Tropen-Hyg.*, 9: 181.

KERR, T. S. 1905a. Kalabarbenlen und ihre Beziehungen zu *Filaria loa* und *diurna*. *Münch. med. Wchnschr.*, 52: 474.

Review of Kerr, T. S., 1904.

KINGSLEY, MARY H. 1897. Travels in West Africa. Congo Français, Corisco and Cameroons. London; Macmillan and Co., 8vo.

Under diseases the author notes "lastly a peculiar abomination, a filaria. I have seen the eyes of natives simply swarming with these filariae. A similar but not identical worm is fairly common on the Ogowé, and is liable to get under the epidermis of any part of the body."

KRAEMER, A. 1899. Die tierischen Schmarotzer des Auges, IV. Die Fadenwürmer (*Filariæ*) des Auges. Gräfe-Samisch Handbuch, II. Theil, X. Band, 10. und 11. Lief., XVIII. Kapitel: 64-87, Figs. 7-9. 182 pp., 17 figs. Rev. *CB. Bakt. u. Par.*, 28: 517-8.

Full record of earlier cases, with unfortunate misprints in names and dates; includes under *F. loa* doubtful cases and also Addario, determined by Grassi as *F. inermis*. Says *F. loa* occurs in eyelids and fingers, though Morton, the authority cited, only gives this as the opinion of Nassau; believes *F. loa* and also the Guinea worm may wander back into tissue of orbit.

KUHNT, H. 1888. Extraction eines Fadenwurms (*Filaria*) aus der Regio macularis des menschlichen Glaskörpers. *Corr.-Bl. allg. ärztl. Ver. Thüringen*, 17: 541-55. Original not seen. Cited thus by Kraemer, 1899.

KUHNT, H. 1892. Extraction eines neuen Entozoon aus dem Glaskörper des Menschen. *Archiv. f. Augenheilk.*, 24: 205-20; 2 figs.

Peculiar small nematode, not identified by Leuckart; not *F. loa*. Removed by operation from the vitreous body.

LACOMPTÉ, C. 1894. Observation d'une *Filaria oculi* dans la chambre antérieure de l'œil d'une Congolaise; extraction de l'entozoaire. *Ann. Soc. méd. de Gand*, 73: 375-86.

Observation brief (pp. 375-77); report on same (pp. 378-86) identical with Van Duyse, 1895, who is also named here as on the commission.

LALLEMANT, L. 1844. Filaria im Auge eines Negers. *Casper's Wchnschr. f. d. ges. Heilkunde*, 1844: 842.

From negro in Rio Janeiro, broke in removal; case regarded as unique, but assigned to Guinea worm.

- LARREY, D. J. 1812. Mémoires de chirurgie militaire et campagnes. Paris; 4 vols. (1812, 1812, 1812, 1817.)
Cites de Lassus (1: 223) on *Filaria loa*. Copied verbatim by Blanchard, 1899.
- LASSUS, DE. Reported by Larry (1812: 223, q. v.).
- LEIDY, JOS. 1877. See Morton, T. G., 1877.
- LESTRILLE, —. Reported by Gervais et van Beneden (1859: 143, q. v.).
- LEUCKART, R. 1863-76. Die menschlichen Parasiten, etc. 2 vols., 8vo, Leipzig.
Full analysis of older cases.
- LEUCKART, R. 1881. Bericht über die wissenschaftlichen Leistungen in der Naturgeschichte der niederen Thiere während der Jahre 1876-1879. *Arch. f. Naturges*, 1877, 2: 397.
Cites Morton (1877), adds case from European on Loango coast, examined worm, and pronounced *F. loa* a good species. Though bearing the date of 1877, and usually quoted as such, this article includes data up to 1879! It was received by the Harvard Library in December, 1881. I adopt this year as the date of the paper.
- LINSCHOTEN, JAN HUYGHEN VAN. 1596. Itinerario. Voyage ofte Schipvaert von Jan Huygen van Linschoten naer Oost ofte Portugaels Indien, etc. t' Amstelredam: Cornelis Claesz; 4to.
This work, of which only three editions are noted here, is often incorrectly cited as giving evidence of the occurrence of *F. loa* in Africa in 1598 (cf. Ward, 1905). This, the original edition, has not the plate supposed to illustrate the extraction of *F. loa*.
- LINSCHOTEN, JAN HUYGHEN VAN. 1610. Histoire de la Navigation de Iean-Hugues de Linscot Hollandois et de son Voyage es Indes Orientales, etc. Amstelredam; Theodore Pierre; 4to.
This later reprint possesses the plate in question.
- LINSCHOTEN, JAN HUYGEN VAN. 1885. The voyage of . . . to the East Indies. From the Old English translation of 1598. The First Book. Edited, in 2 vols., Hakluyt Society, London. (Reprint of edition of 1598.)
The footnotes of this reprint (pp. 46-52) disclose clearly the fictitious character of the plate in question.
- LINSTOW, O. VON. 1900. Ueber die Arten der Blutfilarien des Menschen. *Zool. Anz.* 23: 76-84.
Discusses briefly the form *Filaria diurna* Manson, and the view that this is the larva of *F. loa*.
- LIPPERT, A. B. 1905. Letter cited in Vail, 1905, q. v.
- LOGAN, —. 1895. *Filaria loa*. *Liverpool Med. Chir. Jour.*, 15: 135-42.
Not seen; cited after Penel, 1905a: 157.
- LONEY, W. 1844. Extirpation of Dracunculi from the Eye. *Lancet* (London), 1: 309.
English marine surgeon removed *F. loa* from two natives of west coast of Africa. Description scanty.
- LOOSS, A. 1904. Zur Kenntniss des Baues der *Filaria loa* Guyot. *Zool. Jahrb.*, Abt. 1, *Syst.*, 20: 549-547, 1 pl.
Extended and admirable account of the anatomy, with careful references to earlier work on this phase.
- LOOSS, A. 1905. Von Würmern und Arthropoden hervorgerufene Erkrankungen. *Handbuch der Tropenkrankheiten*, herausgegeben von Dr. C. Mense, 1: 77-209; 54 text figs., Pls. 8, 9.
F. diurna (p. 167); *F. loa* (pp. 177-79). Brief but very complete and correct review of anatomy, life-history, and pathology, so far as known, including account of Calabar swellings, which are likened to those produced by *Sparganium Mansoni*.

- LOPEZ, E. 1891. *Filaria* en la camaria anterior. *Rev. de cien. med.* (Habana), 6: 269.
Not found; cited after Kraemer, 1899.
- LOTA, [F. L.] 1884. [Filaire sous-conjonctival.] In Terrin, L. 1884.
Cited in full in the present paper.
- LUDWIG, H. 1896. *Filaria loa*. *Sitz.-Ber. niederrhein. Ges. f. Nat. u. Heilk.* (Bonn), 1896; natw. Sect., pp. 50-52. (Sitzung 3. Feb., 1896.)
Report of previous papers, including Ludwig and Saemisch (1895). Added data from Manson's and Robertson's cases.
- LUDWIG, H., UND SAEMISCH, TH. 1895. Ueber *Filaria loa* Guyot im Auge des Menschen. *Zeit. f. wiss. Zool.*, 60: 726-40, 1 pl. Rev. in *CB. Bakt. u. Par.*, 1. Abt., 19: 424-5; *Lubarsch-Ost.*, 3: 618; *Zool. Cent.*, 3: 209-10; *Schmidt's Jahrb.*, Bd. 251; *Ann. Ophth. and Otol.* (N. Y.), 5: 1097-8.
Female specimen extracted from beneath conjunctiva of Russian marine officer, whose last trip to west coast of Africa was in 1891. Careful description of anatomy of worm.
- MACNAMARA. 1863. *Filaria papillosa* in the Eye of a Man and the Horse. *Indian Ann. Med. Soc.* (Calcutta).
Briefly noted by Robertson (1894) and others. Cited after Huber (1898). The date is given 1864 by some authors.
- MALGAT, [J.] 1893. Filaire ou dragoneau du corps vitré. *Rec. d'ophtal.* (Paris), (3) 15: 280-3.
Case of man in French Alps; description uncertain, probably filament or artery; certainly not *F. loa*.
- MANSON, SIR PATRICK. 1891. The *Filaria sanguinis hominis major* and *minor*, Two New Species of Haematozoa. *Lancet* (London), 1891, 1: 4-8; 15 figs.
Suggests that *F. loa* when in the eye has "lost its way," and its proper habitat is some part more favorable for release of the embryos into the general circulation. Queries whether the smaller form, later called *F. perstans*, might not be the larva of *F. loa*. Subsequently he assigned this rôle to the larger species, now called *F. diurna*.
- MANSON, SIR PATRICK. 1893. The *Filaria sanguinis hominis* and *Filaria* Disease. Chap. 21 in *Hygiene and Diseases of Warm Climates*, by Andrew Davidson (Edinburg and London), pp. 738-851. Figs. 51-78.
Not on *F. loa*.
- MANSON, SIR PATRICK. 1893. *Diseases of the Skin in Tropical Climates*. Chap. 24 in *Hygiene and Diseases of Warm Climates*, by Andrew Davidson (Edinburg and London), pp. 928-95. Figs. 80-97.
Record of *F. loa* (p. 961) quoted from Morton, 1877. Also case of negro with *F. loa* and later *F. diurna* in blood. Suggests relation.
- MANSON, SIR PATRICK. 1895. See Robertson, D. A. 1895*b*.
- MANSON, SIR PATRICK. 1898. *Tropical Diseases*. London; 8vo.
Two new cases noted briefly. Relation of *F. loa* and *F. diurna* discussed.
- MANSON, SIR PATRICK. 1900. *Ibid.* London; 2d ed., 8vo, 704 pp., 114 illus., 2 col. pls.
Identical in the main with earlier edition, but adds discussion on Calabar swellings.
- MANSON, SIR PATRICK. 1903. Calabar Swellings on the Upper Congo. *Jour. Trop. Med.*, 6: 347-48.
Records eight cases among missionaries, two coming under his own observation. The peculiar geographic range, transient character, irregular recurrence of these swellings, and association with *F. loa*, all point to casual relation. Conjectures the cause as the parturition of *F. loa*; failure to find embryos due to time or incompleteness of observation.

MANSON, SIR PATRICK. 1904. A Note on Dr. Primrose's Paper on Filariasis. *Brit. Med. Jour.*, 1904, 2: 72-3.

Upholds specific distinctness of *F. diurna* from *F. bancrofti*, as against Annett, Dutton, and Elliott. Reports occurrence of *F. diurna* in case of sleeping sickness in Paris at this time.

MAUREL, —. 1868. Recorded as Obs. III by Trucy, 1873 (*q. v.*).

MITCHELL, H. 1859. Report of a Case of a Guinea Worm in the Eye. *Lance* (London), 2: 533-34.

Young negress taken from west coast of Africa to Trinidad in 1834; worm first seen in 1837, again in 1841, 1845; hence at least eleven years in body; had grown from 0.5 in. to 2 in. Felt in body later, but not seen [same worm?].

MONGIN, —. 1770. Observation sur un ver trouvé sous la conjonctive, à Mari-barou, isle Saint-Dominique. *Jour. de méd.* (Paris), 32: 338-39.

Earliest known case, negress of St. Domingo; worm extracted.

MONIEZ, R. 1896. *Traité de parasitologie*. Paris; 8vo, 680 pp.

Short description (p. 351); annotated list sixteen cases. Refers in footnote to Guyon's discovery of plate of 1598 showing operation for removal of eyeworm (cf. Ward, 1905).

MOQUIN-TANDON, A. 1859. *Eléments de zoologie médicale*. Paris; J. B. Baillière et Fils; 12mo, 428 pp., 122 figs. [Title-page date 1860. British Museum stamp date 24 De. 59; also in printed catalogue.]

Brief account, unchanged in later editions and translations.

MORTON, T. G. 1877. Account of a Worm (*Dracunculus* or *Filaria loa*) Removed by a Native Woman from beneath the Conjunctiva of the Eyeball of a Negress at Gaboon, West Africa, with a Brief History of the Parasite and Professor Leidy's Description of the Specimen. *Amer. Jour. Med. Sci.* (2), 74: 113-16.

Specimen sent by Rev. Dr. Nassau; first brought to U. S. A., dried in transit; description scanty; clinical notes by Dr. Nassau valuable, as he had been infected personally. This, and the case of an English trader also mentioned, are the first records of infection among Caucasians.

NAKAIZUMI, Y. 1903. Ueber eine Filaria im Glaskörper des Menschen. *Ophth. Klin.* (Stuttgart), 7: 116-22.

Brief record of foreign body in vitreous humor which, on account of continued movements, the author regarded as a filaria. The suggestion that it was an immature *F. loa* is inadmissible; if any species it is more probably *F. papillosa* or *F. equina*.

NEVE, ARTHUR. 1895. *Filaria loa*. [Letter from mission hospital, Kashmir, Jan. 7, 1895.] *Lancet* (London), 1895, 1: 446.

Reports horse with *F. loa* in anterior chamber of eye. Specimen lost. More probably *F. equina*, common in India.

NORDMANN, ALEX. VON, 1832. *Mikrographische Beiträge zur Naturgeschichte der wirbellosen Thiere*. Berlin; 4to; Heft 1, Erste Abhandl., pp. 1-54.

Gives (p. 7) the history of *Filaria oculi humani*. Small nematode in lens; not *F. loa*.

NORDMANN, A. VON, 1843. Sur les helminthes dans l'œil des animaux supérieurs. (Extrait du Nordmann, 1832.) *Arch. méd. comp.*, 1: 67-113 pl.

Literal translation of Nordmann, 1832.

NORDMANN, A. VON. 1843a. Ueber die Parasiten im Auge der höheren Thiere. *Arch. d. vergl. Med.*, 1: 67.

Cited by Kraemer, 1899. Not found; apparently an unwarranted translation of the periodical name as well as the title of Nordmann, 1843.

NORDMANN ET RAYER. 1843. Helminthes dans l'œil de l'homme. *Ann. d'oculist.*, 9: 136-177.

Reprint of so much of Nordmann (1843) and Rayer (1843) as concerns the human eye, with introduction, footnotes and summary by the editor, Cunier.

OZZARD, A. T. 1903. *Filaria loa*. *Jour. Trop. Med.*, 6: 139. Also corrections by Thompstone, *Ibid.*, 6: 160.

Two males and two females collected by Thompstone in Opobo, Nigeria. Description scanty. Purely anatomical.

PACE, A. 1867. Sopra un nuovo nematode. *Giorn. sci. nat. ed econom.*, 2.

Worm taken from tumor of upper eyelid of boy; named *F. palpebralis* (nec Wilson, 1844). Not *F. loa*, perhaps *F. conjunctivae* Addario (1885, q. v.).

PENEL, R. 1904. Les filaires du sang de l'homme. *CR. sect. méd. et hyg. colonial* (Paris), 199-217.

The autopsy of a Congo negro in Paris showed many adult *F. loa* in the superficial connective tissue of the appendages, none elsewhere. *F. loa* appears in the eye only when young and active. It lives later elsewhere and causes transitory unexplained troubles, or more often none at all.

PENEL, R. 1905. Les filaires du sang de l'homme. *Arch. Parasitol.*, 9: 187-204.

Reprint of Penel, R. 1904.

PENEL, R. 1905a. Les filaires du sang de l'homme. 2me éd., Paris; 163 pp., 20 figs.

Greatly expanded throughout. Includes new chapter on *F. loa*, giving full details of Case 78. Emphasizes the immature condition of the forms taken from the eye, where their occurrence is, moreover, purely accidental.

PICCIRILLI, —. 1879. Del elimintiasi oftalmica. *L'Indipendente*, 1879: 425-30.

Not seen; cited after Parona, *Elmintologia Italiana*. Small structures in anterior chamber; probably not worms.

PICK, L. 1905. Demonstration einer durch Operation gewonnenen *Filaria loa*. *Deutsche med. Wchnschr.*, 31: 1172.

Specimen taken from under conjunctiva in February, 1905. Host lived in Kamerun 1897-98, since then in Germany. No intimation of its presence until day before its removal. "The worm is an intestinal (*sic*) parasite."

PIGAFETTA, FILIPPO. 1598. Vera descriptio regni africani, quod tam ab incolis quam Lusitanis Congus appellatur. Francoforti VV. Richter, and Th. and Io. de Bry.

Falsely cited by various authors as furnishing evidence of the occurrence of *F. loa* in Africa in the sixteenth century (cf. Ward, 1905).

PLEHN, FR. 1898. Die Kamerun-Küste: Studien zur Klimatologie, Physiologie und Pathologie in den Tropen. Berlin; 8vo, 363 pp., 1 chart, 47 text figs.

Observed three cases in Kamerun negroes; a fourth in an English official was not seen personally. According to natives, the worm occurs in the eye of goats and sheep also. Attributes to *F. loa* "probably" also certain fugitive swellings and dermal inflammations about the size of a silver dollar.

PRIMROSE, A. 1903. Filariasis in Man Cured by Removal of the Adult Worms in an Operation for Lymph Scrotum. *Brit. Med. Jour.*, 1903, 2: 1262-5.

Records two cases of *F. loa* in Canada. No examination of blood for embryos.

PRIMROSE, A. 1905. *Idem*. *Canad. Pract. and Rev.* (Toronto), 30: 135-46.

Reprint of Primrose, 1903.

PROUT, W. T. 1902. Filariasis in Sierra Leone. *Brit. Med. Jour.*, 2: 879-81
Rev. CB. Bakt. u. Par., 32 R: 528.

One case *F. loa* in a European; two worms removed; one from eyelid, other from loose skin of penis; patient had lived in Congo; blood swarming with embryo nematodes. First case in Sierra Leone, probably introduced.

QUADRI, A. 1858. (Note dans procès-verbaux de la deuxième section, séance du 15 sept., pp. 153-57, 3 figs.) Congrès d'Ophthal. de Bruxelles, *Compte rendu* (Session de 1857). Paris.

Filaria in vitreous body determined by ophthalmoscope; pronounced by later critics nothing more than persistent hyaloid artery, although confirmed by Della Chiaje.

- RAILLIET, A. 1893. *Traité de zoologie médicale et agricole*; 2me éd., Paris; 1re fascicule.
Brief (p. 529); no new cases or facts.
- RAYER, P. 1843. Note additionnelle sur les vers observés dans l'œil ou dans l'orbite des animaux vertébrés. *Arch. méd. comparée*, 1: 113-54.
Cites 13 cases in all, the last of which concerns a cysticercus. Some others are uncertain also.
- ROBERTSON, D. ARGYLL. 1894. *Filaria loa*. Medical Societies. (Ophthalmological Society. Meeting, Oct. 18, 1894.) *Lancet* (London), 1894, 2: 977-8. Also *Brit. Med. Jour.*, 2: 920-21.
Woman lived eight years in Old Calabar; worm noted in both eyes; removed eight months after return; cites other cases. Discussion by Manson, notes resemblance between embryos of *F. loa* and *F. diurna*.
- ROBERTSON, D. ARGYLL. 1894a. Case of *Filaria loa* in Which the Parasite Was Removed from under the Conjunctiva. *Ophth. Rev.* 13: 329-31. Rev. *CB. Augenheilk.* 1894: 388.
Same case as 1894. Both preliminary to Robertson, 1895b.
- ROBERTSON, D. ARGYLL. 1894b. Cas de *Filaria loa* sous-conjonctivale. *Ann. d'oculist.*, 112: 336.
Literal translation of Robertson, 1894a.
- ROBERTSON, D. A. 1895. A Case of *Filaria loa*. *Ophthal. Rev.* (London), 14: 93-94.
Removal of second specimen from same patient as noted in Robertson, 1894. Preliminary to Robertson, 1895b.
- ROBERTSON, D. A. 1895a. [Translation of *Proc. Ophth. Soc. United Kingdom*, Mar. 14, 1895.] *Ann. d'oculist.*, 113: 277-78.
Translation of Robertson, 1895.
- ROBERTSON, D. A. 1895b. Case of *Filaria loa* in Which the Parasite Was Removed from under the Conjunctiva. *Trans. Ophth. Soc.* (London), 15: 137-67; 2 pls. Rev. in *Arch. Ophth.* (N. Y.), 25: 421.
Records four new cases, reviews old cases, adds note on female *F. loa* from right upper eyelid of same patient and reports by Manson on structure of these specimens and Logan's.
- ROBERTSON, D. ARGYLL. 1895c. Demonstration einer *Filaria loa*. *Ber. Versammel. ophth. Ges.* (Heidelberg), 24: 238. (Pub. at Stuttgart.)
Brief description and exhibit of specimens (two females and one male) from England.
- ROBERTSON, D. ARGYLL. 1897. *Filaria loa*. Ophthalmological Society. *Lancet* (London), 1897, 1: 1744.
Return of his patient to Old Calabar two years previous marked by immediate recurrence of her symptoms in aggravated form. Itching behind the eyes and swellings in the arms were most prominent and said to be a most universal in Gaboon. Return to England, but no relief. No embryos in blood, excreta, saliva, or mucus.
- ROBERTSON, D. ARGYLL. 1897a. [Quoted on *F. loa* in London letter of July 2.] *Med. Rec.* (N. Y.), 52: 104.
Brief abstract of Robertson, 1897.
- ROBERTSON, D. A. 1904. Letter quoted by Habershon, 1904 (*q. v.*).
- ROTH, FELIX. 1896. *Filaria loa*. *Lancet* (London), Rev. in *CB. Bakt. u. Par.*, 19: 790-91.
Native girl on Niger coast, West Africa; worm not extracted. Other cases in same village. This specimen in eyelid wandered across to other eyelid.
- ROULIN. 1832. Dragoneau. *Arch. gén. de méd.*, 30: 573.
This reference is given as above by Blanchard (1899) and others. Guyon (1864) says it is wrongly attributed to Clot-Bey (*q. v.*) in the review where it was published. I can find no trace of a similar article by Roulin in this volume or elsewhere.

ROUX, FERNAND. 1888. *Traité pratique des maladies des pays chauds*. Paris: G. Steinhilf; 3 vols.

Brief (3: 552); no new cases; gives *F. lachrymalis* as synonym.

SAMBON, L. W. 1902. Remarks on the Individuality of *Filaria diurna*. *Jour. Trop. Med.*, 5: 381-84.

Careful critique of Annett, Dutton, and Elliott's view of the identity of *F. diurna* with *F. bancrofti*. Some difficulties due to mixed infections, others to incomplete evidence. No other embryo in West Africa which can belong to *F. loa*. Known facts accord with probable life-history as taken from other species of *Filaria*.

SAMBON, L. W. 1903. (Continuation of 1902.) *Jour. Trop. Med.*, 6: 26. Trans. in *Caducée*, 3: 305.

Annett, Dutton, and Elliott's suggestion of a diurnal mosquito as host for *F. diurna* fatal to their theory of identity. Manson's suggestion of Mangrove Flies more probable; certainly to be found among Tabanidae.

SANTOS, CHRISTOVO JOSE DOS. 1833. Case recorded in Sigaud, 1844 (*q. v.*).

SANTOS-FERNANDEZ, D. J. 1879. *Filaria* en al cuerpo vitreo. *Cron. méd-quir. de la Habana*, 5: 436-38.

Not found; cited from Surgeon General's Catalogue. "Twice found nematodes in vitreous humor" (Yarr, 1899).

SANTOS-FERNANDEZ, D. J. 1882. *Cron. méd-quir. Habana*, 8: 116.

Cited thus by Kraemer, 1899. The page given is incorrect, and I could not find any such paper or note in Vol. 8.

SCHEUBE, B. 1900. *Die Krankheiten der warmen Länder*. Jena: G. Fischer, 2d Aufl., 661 pp., 7 pls., 5 charts, 30 text figs.

Says (p. 492) *F. loa* can be in anterior chamber, and is probable cause of Calabar swellings.

SCHEUBE, B. 1903. *The Diseases of Warm Countries*. Translated from the German by Pauline Falcke; edited by James Cantlie. London: John Bale, Sons. 2d ed.; 594 pp., 7 and 12 pl., 58 text figs.

F. loa (p. 441); says Manson has relinquished the view that *F. diurna* is the larval form corresponding to this adult.

SCHÖLER. 1875. (Demonstration.) *Berlin. klin. Wchnschr.*, 12: 682 (13: 8, discussion).

Before Berlin Medical Society; woman with living nematode 12-15 mm. long spirally rolled and actively moving in lens. Virchow examined carefully. Interpreted by later critics as persistent hyaloid artery.

SERMON, G. 1872. Case of *Filaria oculi* Occurring in Practice; Operation and Recovery. *Canada Med. Rec.* (Montreal), 1: 173.

The patient was a bay mare! The species certainly not *F. loa*.

SIEBOLD, C. TH. 1839. Bericht über die Leistungen im Gebiet des Helminthologie während des Jahres, 1838. *Arch. f. Naturg.*, 1839, 2: 152.

Brief reference to case of Guyot [Guyon?]; also to Clot. Moniez (1896) says that both names are wrong in this review.

SIGAUD, J. F. 1844. *Du climat et des maladies du Brésil, ou statistique médicale de cet empire*. Paris, 8vo.

A filaria (p. 135) in the orbit, behind the sclerotic; in a negress of Rio Janeiro. May have been a Guinea worm, and not a *loa*.

STELLWAG VON CARION, CARL. 1858. *Die Ophthalmologie*. Erlangen, 2 vols.

Quoted by Kraemer, 1889, as a case of Guinea worm in the orbit; account very brief; no cases or data given; more probably referable to *F. loa*.

- STOSSICH, M. 1897. Filarie e Spiroptere: Lavoro monografico. *Boll. Soc. Adriat.*, 18: 13-162. *Rev. Zool. Centr.*, 5: 124; *Jour. Roy. Mic. Soc.*, 1898: 63. Brief taxonomic description (p. 21); few citations.
- SUPINO, F. 1900. Sopra una Filaria dell'occhio umano. *Rend. Acc. Lincei* (5), 9: 85-91. 3 figs.
Not *F. loa*. Specimen from Grassi, same as Addario's (1885) *F. conjunctivae*.
- TERRIN, L. 1884. Étude sur le cysticerque de l'œil. Thèse, Fac. de méd. Montpellier, No. 78.
F. loa (pp. 46-48) as Obs. V. par M. Lota.
- TEXIER, —. 1904. A propos de la filariose. *Ann. d'hyg. et de méd. coloniales*, 1904: 102.
Not seen; according to Penel (1905a: 82) he records a new case which manifested Calabar swellings and had *F. diurna* in the blood.
- THOMPSTONE, S. W. 1899. Calabar Swellings. [Letter with editorial additions.] *Jour. Trop. Med.* (London), 2: 89-90.
Discusses fugitive swellings at Old Calabar. Editor notes similar trouble in Robertson's patient with *F. loa*, but only "since her return home."
- TRUCY, CH. 1873. Remarques sur la Filaire de Médine et en particulier sur son traitement. Thèse, Fac. de méd., Montpellier, No. 22, 4to; 42 pp.
Regards *F. loa* as identical with Guinea worm (p. 8), and cites one case by Maurel in Gaboon who extracted worm in 1868. Complete recovery.
- TURNBULL, C. S. 1878. Filaria in the Eye. *Med. and Surg. Reporter* (Phila.), 39: 351-55.
Only brief reference to previous cases of *F. loa*. Case observed was in eye of horse.
- VAIL, D. T. 1905. *Filaria loa*. African Correspondence. *Lancet-Clinic* (Cincinnati), 55: 733-34.
Prints with comments letter from Dr. A. B. Lippert in Kamerun who has removed "dozens" of the *loa* recently, and thinks worm ultimately dies in body and is absorbed. A motionless filaria under conjunctiva appeared nodular and proved to be adherent to surrounding tissue. Since then has had a number of cases and has learned to recognize nodules under conjunctiva as dead filaria in fibrous sheath.
- WARD, H. B. 1902. A Record of the Occurrence of *Filaria loa*, a Human Parasite New to the United States. *Science, N. S.*, 16: 350.
Brief announcement of the specimen of Milroy and of the reading of this paper. No data given.
- WARD, H. B. 1903. Nematoda. Wood's *Reference Handbook of Medical Sciences*. Rev. ed., 6: 205-25.
Reference (p. 211) to case of Milroy, and figure of posterior end of this specimen, specules incorrectly reproduced.
- WARD, HENRY B. 1905. The Earliest Record of *Filaria loa*. *Zool. Annalen*, 1: 376-84. 1 fig.
Shows that the illustration cited from records of early voyages as evidence of the occurrence of *F. loa* is a fancy picture and cannot be interpreted in the manner suggested.
- WARD, HENRY B. 1905a. Studies on Human Parasites in North America. I. *Filaria loa*. (*Studies from the Zoological Laboratory, The University of Nebraska*, No. 63.) *Jour. Infect. Dis.*, 1906, 3, p. 37.
The present paper.
- WILSON, F. M. 1890. Specimens of *Filaria oculi humani*. *Trans. Amer. Ophth. Soc.* (Hartford), 5: 727-29.
Incompletely cited by Blanchard, 1899; missionary at Benita (Gaboon), West Africa, says at intervals all natives feel worms in different parts; extract them from eye only. She had one removed at Basel, (Switzerland), February, 1889, from left upper eyelid; one in Bridgeport, Conn., November, 1899, from

right upper eyelid; one in Clifton Springs, N. Y., February, 1890, from beneath skin of back; and broken one July 1890, from right upper eyelid. "So far as I have been able to obtain the evidence from the missionaries themselves, these filariæ are more common in the cellular tissue than in the eyeball. From the literature we should infer the opposite."

WURTZ, R. 1904. Présentation d'une *Filaria loa*. *Soc. méd. hyg. trop.*, séance 20 jan.

Not seen; cited after Wurtz and Clerc, 1905.

WURTZ, R., ET CLERC, A. 1904. Eosinophilie intense provoquée par la *Filaria loa*. *CR. Soc. Biol.* (Paris), 55: 1704-5.

Young French girl in Congo with Calabar swellings and *F. loa* had no embryos in blood, but intense eosinophilia; latter known for genus *Filaria* but not noted heretofore in *F. loa*.

WURTZ, R., ET CLERC, A. 1905. Nouvelle observation de *Filaria loa*. Considérations sur l'hématologie des filarioses. *Arch. méd. exp.* (Paris), 17: 260-66.

Same patient as above returned to France in June, 1903. In January, 1904, worm extracted from eye. Eosinophilia somewhat reduced; returned later. Extreme symptoms and continuance indicate that several parasites are present. Discussion of parasite Calabar swellings and eosinophilia in helminthiasis.

YARR, M. T. 1899. The Filariæ of the Eye. *Jour. Trop. Med.*, 1: 176-79.

Native name of *loa* means simply "worm." Good review of previous knowledge. No new cases. Records the conjecture of Manson that the cases from the West Indies, also that of Barkan (1876), concern the adult of *F. Demarquaii* and not *F. loa*.

ZIEMANN, HANS. 1905. Beitrag zur Filariakrankheit der Menschen und Thiere in den Tropen. *Deutsche med. Wchnshr.*, 31: 420-24.

F. loa (p. 421) increasing in that region. Sees in *F. perstans* in blood embryos of *F. loa* and unites *F. dirurna* to *F. bancrofti*. Not every case with *F. perstans* in blood and Calabar swellings has had *F. loa* in eye. Distribution of microfilariae in body very irregular. Data on other species, therapy, etc.

ADDENDUM.—The paper of Penel (1905a) reached me after the MS. of this paper had left my hands and the brief notes which could be introduced into the proof do not do justice to the important results presented in this publication. The author has anticipated my explanation that the eye filariæ are erratic and immature wandering forms, as set forth in the preceeding pages.

H. B. W.

DISSEMINATED BLASTOMYCOSIS.

REPORT OF A CASE INVOLVING THE LUNGS, LUMBAR
VERTEBRÆ, AND SUBCUTANEOUS TISSUES, WITH
MULTIPLE ABSCESES AND FISTULÆ,
AND EXTENSIVE AMYLOID
DEGENERATION.*

PETER BASSOE.

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THE CLINICAL RECORD.†

G. S., a boy 17 years old, entered the service of Dr. Senn in the Presbyterian Hospital on July 26, 1904. Both parents are living and in good health. A sister nine years old is living and well. Four brothers and sisters died during infancy or early childhood. No history of any form of tuberculous trouble in any member of the family can be obtained from the patient. The patient is a native of Chicago, and has lived in the neighborhood of Haymarket Square, Chicago, doing any kind of work he might pick up. He had measles and mumps in childhood, later gonorrhea. The history of the ailment for which he sought admittance to the hospital reads as follows: "In March of this year (1904) he slipped and fell, striking on the right shoulder. The next day the shoulder was lame and he went to a doctor, who told him that he had a sprain, and put on a plaster dressing. There was at that time some swelling and considerable pain which later partly disappeared. Since April the swelling has been stationary and painless except on pressure. The patient states that for some time previous to this trouble he had pain throughout the whole lumbar region, and a gnawing sensation in the upper part of the right lung, and that he has lost 30 pounds in weight. He coughs considerably and has night-sweats." The temperature on the day of admittance was 99.8°, pulse 100, respiration 20. On the same day (July 26, 1904), a trocar was passed into a large abscess over the right scapula and about one ounce of thick, slightly blood-stained pus was evacuated. Iodoform emulsion was injected and the wound sealed with collodion. The urine was normal. During the following week the temperature rarely exceeded 100° (maximum 100.2°). On August 2, the abscess over the scapula was opened by a transverse incision 3.5 inches long, scraped out, and packed with iodoform gauze. During the week following the temperature on several occasions reached 102°. August 9, a large abscess in the right lumbar region was freely incised and one pint of pus evacuated. During the following two months there was a daily rise in temperature to 100° or 101°, the pulse commonly fluctuating between 64 and 112. The respirations were not accelerated. September 1 a blood count revealed 4,180,000 erythrocytes and 19,500 leucocytes. Blastomycetes were repeatedly found in smears of pus from the various subcutaneous abscesses. Later they were also found in the sputum, never in the feces. Pure cultures of blastomycetes from the pus were obtained by

* Received for publication December 28, 1905.

† For permission to quote from the Hospital records, I am indebted to Dr. E. J. Senn.

Dr. R. H. Goheen, who also successfully inoculated guinea-pigs and recovered the organisms from them.*

The patient was discharged improved on November 15 with a diagnosis of blastomycosis, and readmitted on December 18, 1904. The temperature during the first two weeks generally ran between 100° and 103°. Nausea and diarrhea were present at times. On January 3, 1905, abscesses in the right scapular and in the lumbar region were incised. The irregular fever continued. A leucocyte count of January 25 was 1,760, hemoglobin 50 per cent. He frequently complained of pain in the back and right shoulder and of nausea with occasional vomiting. In May repeated examination of the urine revealed a large quantity of albumin with hyaline and granular casts; 1,600 c.c. of specific gravity 1,012 were voided in 24 hours. The reaction was alkaline. Blood pigment and red cells were found. During the last two months of the patient's life diarrhea was constant and gradually increased in severity. The limbs became edematous and painful. Abdominal pain was frequent. The irregular fever persisted and the patient gradually became weak. The temperature was subnormal the last four days of the patient's life. Death occurred on June 27, 1905.

SUMMARY OF THE RESULTS OF THE GROSS AND MICROSCOPIC EXAMINATION AFTER DEATH.

Chronic subcutaneous blastomycetic abscesses in right scapular region and in loin; abscess and sinus walls made up of vascular granulation tissue rich in polymorphonuclear leucocytes, mast cells, and blastomycetes. Blastomycotic caries of fourth and fifth lumbar vertebræ, with bilateral psoas abscesses. Disseminated blastomycotic broncho-pneumonic foci in both lungs. Areas of necrosis with Langerhans giant cells in the mediastinal glands. Amyloid degeneration of spleen, liver, adrenals, retroperitoneal, mesenteric and mediastinal lymph nodes, kidneys and colon.† Bilateral fibrinous pleuritis, and mild sero fibrinous peritonitis. Chronic parenchymatous nephritis. Atrophy of the heart. Pulmonary edema. Edema of feet and thighs. Tigrolysis of ganglion cells of cerebral cortex and ventral horns of cord (only upper cervical portions of latter examined).

THE NECROPSY RECORD.

The necropsy was held by the writer six hours after death. The following record was made:

The body is that of an emaciated young man with long, slim neck and slender bony development. Rigor mortis is present. The lower extremities are flexed at the knees at an angle of about 145 degrees, and cannot be extended without considerable force. There is edema of both feet and slight edema on the inner aspect of both thighs. Below the spine of the right scapula is a defect in the skin 5.5 cm. from above downward,

* Dr. Goheen is now abroad, and I have been unable to learn further details of his work. His records and specimens unfortunately are not now available.

† Mentioned in order of severity; slight in colon.

and 4 cm. from side to side. The inner border is 4.5 cm. to the right of the median line. At the inner border of the scapula are two openings each about 1 cm. long. Through the lower opening a probe passes upward and inward for a distance of 1 cm. before meeting any resistance. Through the upper opening it passes upward to the inner border of the scapula just beneath the skin, for a distance of 6 cm. In laying the upper sinus open, it is found to have a smooth shiny lining, with a small amount of liquid secretion. The lower sinus has a similar lining; it ends just behind the clavicle. There is apparently no connection with the chest cavity. There is another opening on a level with the crest of the ileum, and 6 cm. to the right of the median line of the back. Through this sinus a probe is passed outward for a distance of 7 cm. The sinus ends blindly at the crest of the ileum, in the subcutaneous tissue just above the crest. From the middle of the sinus a narrow opening passes inward, and a probe may be passed 5.5 cm. in the direction of the abdominal cavity. Six cm. to the left of the median line of the back, and 2 cm. lower than the above, is another smaller opening discharging whitish pus. Through this opening a probe passes 5 cm. in the direction of the abdominal cavity.

Very little subcutaneous fat is present.

The peritoneal cavity contains about 100 c.c. of turbid, grayish fluid, rich in fibrinous flakes. The peritoneal surfaces are smooth, and are not hyperemic anywhere. There are no abnormal adhesions. The diaphragm reaches to the fourth interspace on each side.

The left pleural cavity is free from adhesions, and contains about 200 c.c. of cloudy, yellowish fluid. The right pleural cavity is partly obliterated by fibrous adhesions, and contains about 200 c.c. of fluid which is more cloudy than that of the left side.

Each lateral lobe of the thyroid measures 3.5x1.5 cm. The cut surfaces are uniformly pale and gelatinous.

The cervical lymph nodes are not enlarged. The nodes in the mediastinum are moderately enlarged, measuring 1.5 cm. in length, and whitish nodules are seen on the cut surfaces. The tracheo-bronchial nodes are of similar size, and are anthracotic, but contain no nodules.

The larynx and trachea have a smooth and pale lining.

The left lung weighs 230 gms. The pleura is smooth and bluish gray. In the surfaces of both lobes numerous shotlike elevations are seen. On palpation both lobes are found to be studded with hard nodules. On the external surface the elevations are yellowish in the middle, surrounded by a bluish-red zone. On section the nodules are also seen to possess a grayish center surrounded by a hyperemic zone. Grayish-white pus can be expressed from the center of many of the nodules. The lung tissue between the nodules crepitates and contains a considerable amount of watery frothy fluid.

The right lung is larger than the left. It weighs 420 gms. All lobes are studded externally by nodules, that average about 3mm. in diameter (Fig. 1). The cut surfaces of the upper and middle lobes resemble those of the left lung. The lower lobe contains more blood and frothy fluid, and the large bronchi contain bloody mucus.

The heart is small, weighing 180 gms. The epicardium is smooth. The tricuspid opening admits six finger-tips, the mitral four. The endocardium shows no changes. The myocardium is uniformly light grayish-red.

The aorta is smooth.

The spleen weighs 220 gms. It is firm, the capsule tense, the surface shiny. The cut surface is of a translucent, grayish-red color, the Malphigian bodies being large, transparent, and gray. On treatment with Lugol's solution, a typical amyloid reaction is obtained.

The tongue, pharynx, and esophagus are smooth.

The stomach is small, measuring 15 cm. from side to side and 5 cm. from above downward. The lining is smooth with numerous minute ecchymotic spots. The rugæ are prominent.

The small intestines are smooth, the mucous membrane thin and the lymphatic structures small.

The large intestines also have a smooth lining.

The liver weighs 1,550 gms. It is smooth externally and firm. The cut surface has a smooth, glistening, bacony appearance. The lobular markings are fairly distinct. The central portions are light grayish-brown; the outer portions are pale gray. No nodules are seen. On treatment with Lugol's solution typical amyloid reaction is obtained.

The gall-bladder is rather small, and contains thick, fluid bile.

The pancreas weighs 90 gms. It is rather firm and pale. The lobular markings are distinct.

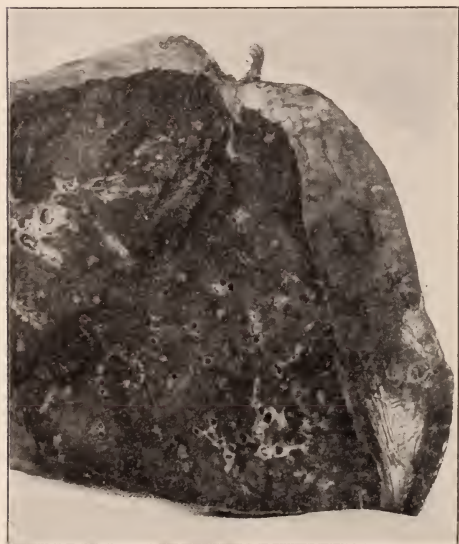


FIG. 1.—Right lung, showing nodules on cut and external surfaces.

The adrenals are of normal size. Their cortical portion is rather thick and glistening, and gives the amyloid reaction with iodine. A few pin-head-sized grayish nodules may be seen in the surfaces of both organs.

The kidneys together weigh 340 gms. The capsule strips readily, leaving a pale, gray surface, on which the stellate veins are prominent. The cut surface is pale gray with indistinct markings; numerous vessels are plainly seen. The cortex is 8 mm. thick; the pelvis is smooth. Both kidneys answer the same description.

The ureters are of normal caliber.

The urinary bladder has a smooth and pale lining.

The prostate is rather small. The cut surface is uniformly white. The seminal vesicles are not altered.

The testicles are rather small. The cut surface is pale gray.

The periaortic lymph nodes are small, the largest measuring 1 cm. in length. They are pale gray on cut sections.

The mesenteric nodes are slightly enlarged, measuring about 1 cm. in length.

On examining the lumbar vertebræ, two openings communicating with the sinuses described in the soft parts of the back are found to lead into necrotic bone tissue. The

necrosis has involved the adjacent parts of the body of the fourth and fifth lumbar vertebræ, and partly destroyed the intervertebral disk, which is replaced by grayish-white, soft necrotic tissue. A considerable amount of grayish pus is present and is found to have burrowed along the sheaths of both psoas muscles, forming suppurating sinuses lined by whitish tissue resembling that lining the subcutaneous sinuses. The pus has burrowed downward to the trochanters, but here both sinuses end blindly. No changes are found in other parts of the spine, or in the right scapula underlying the ulcerated skin described.

The right knee-joint and lower part of the right femur show no changes.

The skull, scalp and dura mater show no changes.

The brain shows no external change. Only the upper part of the spinal cord was removed.

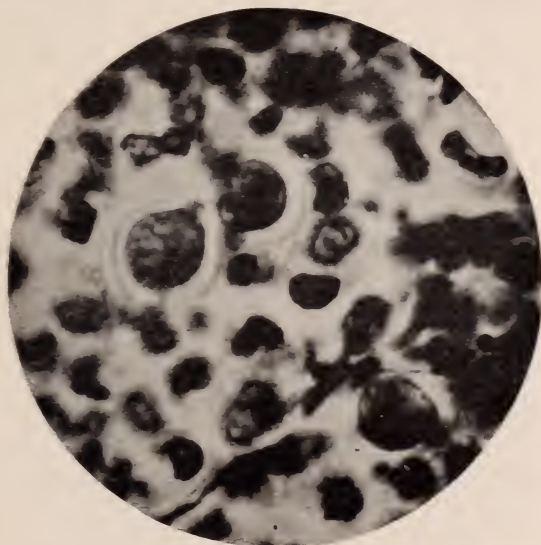


FIG. 2.—Photomicrograph. About $\times 1000$. From the lining of the sinus over the right scapula. Two organisms are seen. Polychrome blue stain.

STUDY OF SMEARS AND CULTURES.

The efforts to cultivate blastomycetes failed, though numerous such organisms were seen in cover-glass preparations of bronchial mucus, of pus from the carious vertebræ, and of pus from the sinuses about the shoulder and loin. Two sets of pipettes were filled with material from the autopsy, and smears and cultures were made independently by Dr. S. A. Berg, and myself. In the main similar colonies were obtained by both of us, and the work of identification of the organisms from both sets was finally carried out by Dr. Berg.

Pericardial fluid.—Smears show no organisms. Cultures sterile.

Right pleural fluid.—Endothelial cells containing fat globules and fatty acid crystals in the smears; no organisms. Colon bacillus in the cultures.

Left pleural fluid.—Smears show cocci; polymorphonuclear cells. Cultures gave *Staphylococcus aureus* and *albus*.

Peritoneal fluid.—Short bacilli in the smears also endothelial cells with fat globules. Colon bacillus in cultures.

Heart's blood.—The colon bacillus and a streptococcus developed late on some of the tubes and plates.

Bile.—Smears and cultures; no organisms.

Bronchial secretion.—Blastomycetes in smears; also cocci. *Staphylococcus aureus* in cultures.

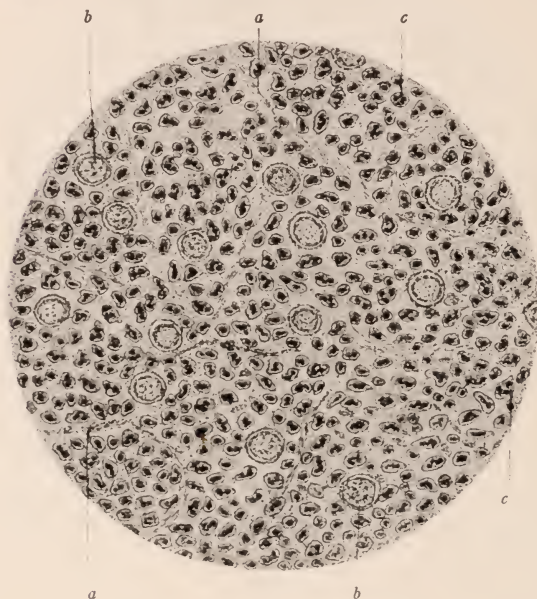


FIG. 3.—Drawing from a nodule in the lung. *a*, Alveolar walls; *b*, Blastomycetes; *c*, Polymorphonuclear cells.

HISTOLOGICAL EXAMINATION.*

Subcutaneous lesions.—Pieces from the ulcerated tissue overlying the scapula were fixed in formalin and stained with hematoxylin-eosin and polychrome blue. There is a large amount of vascular granulation tissue of usual appearance. Many mast cells are seen. Blastomycetes are very abundant, and in two places they are seen within the lumina of blood-vessels. The organisms are of typical appearance, spherical with double contour (Fig. 2). They contain a variable number of large granules stained blue or brownish with polychrome blue. The central portion of the organism as a rule is homogeneous. Several budding forms are seen. In places the granulation tissue also contains numerous long slender bacilli and clusters of cocci.

Sections from the lining of one of the sinuses in the loin show muscle lined by granulation tissue, in which a few blastomycetes are seen. The endothelial cells are seen. The endothelial cells of the vessels are tall. Polymorphonuclear cells and cocci are abundant. A few bacilli are seen.

* When not otherwise stated fixation by Zenker's fluid and 95 per cent alcohol has been used, and staining by hematoxylin-eosin, polychrome methylene blue, and Loeffler's alkaline methylene blue.

Smears of pus from sinus in left loin showed blastomycetes, many of which are budding. Cultures gave the colon bacillus.

Lung emulsion.—Blastomycetes and cocci in smears. Cultures gave *Staphylococcus aureus* and colon bacillus.

Spleen emulsion.—No organisms in smears. Cultures showed *B. pyocyaneus* (contamination?).

Liver emulsion.—Smears and cultures sterile.

Kidney emulsion.—Smears showed no organisms. Cultures gave *B. pyocyaneus* and a pigment-producing bacillus (contamination?).

Lungs.—Sections were made from many portions of both lungs. In the nodular areas the alveoli are filled with inflammatory cells, among them many polymorphonuclears. In the central portion of the areas the alveolar outlines are generally lost, and in places necrosis has taken place. In the outer portions the alveolar outlines are distinct, and the alveoli are chiefly filled with desquamated epithelial cells, leucocytes, and red cells. There are also extensive hemorrhages in the interior of the nodules. Blastomycetes are abundant (Fig. 3) in all parts of the consolidated areas, and occasionally show budding. Many bronchi are filled with a detritus in which numerous large bacilli, many cocci, and occasionally blastomycetes, are found. In the walls of such bronchi mast cells are plentiful; such cells are also occasionally seen about the vessels. In a large number of sections examined, only one giant cell was found. This is located in a partly necrotic nodule, and is of the Langerhans type. No tubercle bacilli are found in sections stained by carbolfuchsin in the usual manner.

Lumbar vertebræ.—A piece which included the intervertebral disc between the two affected vertebræ was fixed in Zenker's fluid, and subsequently decalcified with nitric acid. The cartilage was partly destroyed and covered by a

fibrinous exudate in which pieces of necrotic cartilage and bone are embedded. In the meshes of this exudate blastomycetes are extremely abundant, more so than in any other part of the body from which sections were made (Fig. 4). Numerous blastomycetes are also seen throughout the spongy bone, in one instance within a giant cell (see Fig. 5). In this region the cells of the spongy substance are much more densely packed than in the interior, and the proportion of polymorphonuclear cells is greater.

Lymph nodes.—a) Mediastinal. In sections from one of these, areas of necrosis are seen. A few giant cells of the Langerhans type, plasma cells, and occasionally mast cells, are found. No blastomycetes are seen. Several sections were stained for tubercle bacilli with negative results. Portions of the node are affected by amyloid degeneration.

b) Mesenteric. Here the amyloid change is more marked. Numerous polymorphonuclear cells are seen among the lymphoid cells. There are no areas of necrosis, and no blastomycetes are seen.

c) Retroperitoneal. The amyloid degeneration is considerably more advanced

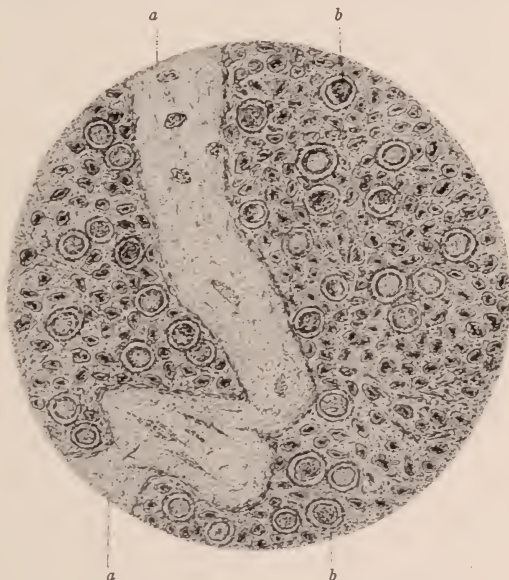


FIG. 4.—Drawing from section of lumbar vertebra. a, Necrotic bone; b, Blastomycetes.

than in the mesenteric nodes. Polymorphonuclear cells are very abundant; there is proliferation of endothelial cells, and numerous mast cells are seen. No blastomycetes are found in a large number of sections examined from various lymph nodes. Bacilli are present in sections stained with methylene blue.

In the *liver*, and in the *spleen* even more so, the amyloid degeneration is marked. The spleen is a typical "sago" spleen.

The *adrenals*, particularly the middle zone of the cortex, are distinctly amyloid.

In the *kidney* most glomeruli present slight amyloid change in their capillaries. The convoluted tubules, as a rule, are slightly dilated, and contain granular detritus;

their epithelial cells in many places are degenerated. There is no increase in interstitial tissue.

The digestive tract.—The tongue and stomach are not materially altered. In the ileum there is extensive necrosis of the mucous membrane. Numerous large bacilli are seen, but leucocytes are very scarce. The walls of many of the small vessels in the mucosa are thickened and homogeneous. In the colon the change is more marked.

There are no noteworthy changes in the *thyroid*, *myocardium*, *prostate*, and *testicle*.

The nervous system.—In Nissl specimens from the paracentral lobules well-marked tigrolysis is present. In the Betz cells the tigroid appears as if finely pulverized in the center of the cells and

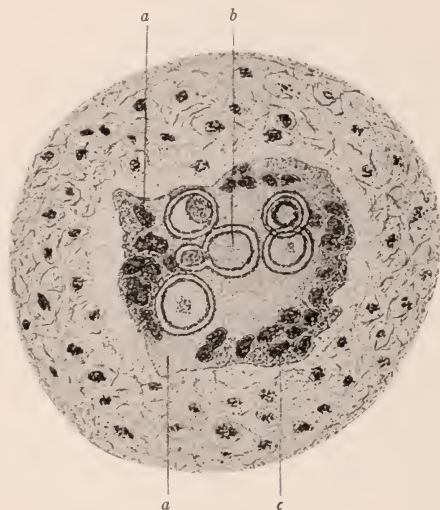


FIG. 5.—Drawing from section of lumbar vertebra showing a giant cell. *a*, Cytoplasm of giant cell; *b*, Blastomycetes (one budding); *c*, Nuclei of giant cell.

as coarse granules along the periphery. Distinct Nissl bodies are found in the dendrites only. The nucleus is frequently peripherally located with ill-defined outline and chromatin network. The dendrites are visible for a long distance. Tigrolysis is also seen in the pyramidal cells of the frontal lobe, and to a lesser extent in the ventral horn cells.

REMARKS.

This is the fifth case of general infection with blastomycetes to be reported from Chicago since 1902.

The first Chicago case was that of Walker and Montgomery¹ in which primary blastomycetic dermatitis was followed by general dissemination which at the necropsy was mistaken for miliary tuberculosis.

¹*Jour. Am. Med. Assoc.*, 1902, 38, p. 867.

In the second case, that of Ormsby and Miller,¹ the lungs appear to have been primarily attacked. At the autopsy lesions were also found in the skin, subcutaneous tissues, liver, spleen, kidney, pancreas, and trachea. Amyloid changes in the kidney are described. A further study of the organisms in this case is found in an article by Otis and Evans.²

In 1904 Cleary³ reported a case in which also the lungs appear to have been primarily involved. Other lesions were found in the cervical nodes, liver, kidneys, spleen, myocardium, and adrenals. There was amyloid degeneration of the spleen, kidneys and adrenals.

In 1905 Eisendrath and Ormsby⁴ reported the case of a man, 33 years old and still living, in whom pulmonary symptoms existed for four months prior to the appearance of cutaneous and subcutaneous lesions. Blastomycetes were found in the sputum. It is a point of great interest that in this patient symptoms of spondylitis recently developed.

The present and fifth Chicago case is very likely also one of primary infection of the lungs, although the history of the onset is not clear. It is the first one in which spondylitis due to blastomycetes, with psoas abscess, has been demonstrated postmortem.

In addition to these five cases the writer knows of several which have been observed in Chicago or vicinity within the past year, and hopes they will soon be placed on record in as detailed a form as possible. A complete case report of generalized blastomycosis does not yet exist. Either the history or the symptomatology, or the microbiologic work during life or after death, or the details of the necropsy, have been more or less defective. Careful recording of details in many cases will be necessary before the clinical and anatomical "textbook" picture of blastomycosis can be drawn.

As yet the organisms concerned have not been placed definitely and it is not now possible to differentiate between the essential and the non-essential in their variable biologic characteristics. For this reason no attempt is made at this time to correlate these five

¹ *Jour. of Cut. Dis.*, 1903, 21, p. 121. ² *Jour. Am. Med. Assoc.*, 1903, 41, p. 1075.

³ *Trans. Chicago Path. Soc.*, 1904, 6, p. 105; also *Medicine*, 1904, 19, p. 818

⁴ *Jour. Am. Med. Assoc.*, 1905, 45, p. 1045.

Chicago cases—all of which apparently are due to very similar organisms—and the few similar cases reported elsewhere. Very likely the previous recorders of cases from Chicago are right when they consider the case reported in Germany by Busse¹ and Buschke² to belong to the same class. The only difference between the organism from this case and those from the Chicago cases seems to be that the former did not produce aerial hyphæ. With our present insufficient botanical knowledge it also is impossible to give an opinion on the relation of these cases to the 13 cases which recently have been collected by Ophüls³ under the title of "coccidioidal granuloma." Besides many new ones from California, Ophüls includes the well-known and frequently discussed cases of Posadas and Wernicke,⁴ Rixford and Gilchrist,⁵ and that of Ophüls and Moffitt.⁶ In all of these cases the organisms are characterized by the absence of budding during their development in the tissues and by their multiplication by endosporulation. Clinically the cases very much resemble the Chicago cases and like the latter they also resemble tuberculosis. Ophüls makes the statement that in infection with blastomycetes the primary focus is always in the skin, whereas in the so-called coccidioidal granuloma it is just as likely to be found elsewhere. This point loses its value when we analyze the five Chicago cases, in only one of which (that of Walker and Montgomery) was the primary focus in the skin. Skin lesions were found in six of Ophüls' 13 cases and considered primary in four cases. Bone lesions, frequently multiple, and strongly resembling tuberculous caries, were found in six of the cases. Multiple bone lesions also existed in the Busse-Buschke case.⁷

I have used the term "blastomycosis" with considerable misgiving, as I am aware that there is a tendency among botanists to designate as "blastomycetes" the budding forms of any fungi which

¹ *Virch. Archiv*, 140, p. 23, and 144, p. 360.

² Volkmann's *Samml. klin. Vorträge*, 1898, 218; also *Verhandl. d. Deutsch. dermat. Gesellsch.*, 1890, p. 181.

³ *Jour. Am. Med. Assoc.*, 1905, 45, p. 1201. ⁴ *Centralbl. f. Bakt.*, 1892, 12, p. 859.

⁵ *Johns Hopkins Hospital Reports*, 1896, 1, p. 209.

⁶ *Phil. Med. Jour.*, 1900, 5, p. 1471.

⁷ A case of myxoma-like tumor in the thigh and deep abscesses due to a yeastlike organism is reported by Curtis (*Ann. de l'Inst. Pasteur*, 1896, 10, p. 449). This case and all other cases of infection with blastomycetes and allied organisms reported up to 1900 are abstracted and thoroughly discussed in the monograph by Ricketts "Oidiomycosis of the Skin and its Fungi," *Jour. Med., Res.* 1901, 1, p. 373.

otherwise may not at all belong together. For the present the term "blastomycosis" is used for lack of a better one and in order to emphasize the fact, so important from a medical standpoint, that we are dealing here with an infection by organisms which bud in the tissues of the human body.

THE RÔLE OF PHAGOCYTOSIS IN THE ANTHRACIDAL ACTION OF DOG BLOOD.*

LUDVIG HEKTOEN.

(From the Memorial Institute for Infectious Diseases, Chicago.)

INTRODUCTION.

THE demonstration by Wright and Douglas¹ of the exact interaction between serum and cells in phagocytosis makes it feasible to study the part played by leucocytes in the destruction of certain bacteria *in vitro*. The pathogenic bacteria most suitable for this purpose are such as are not destroyed by lysins in the serum, e. g., streptococci, pneumococci, and anthrax bacilli (especially so far as dog blood is concerned). The mechanisms of invasion and of healing in infections by these organisms present many difficult problems in the efforts at solution of which special attention must be given to the leucocytes, because of the prominent part played by them in the reactions of the infected body. G. F. Ruediger² has shown that opsonin and leucocytes are essential to destruction of streptococci by human blood, and in this communication I propose to trace the rôle of phagocytosis in the anthracidal action of dog blood.

THE OPSONIN FOR ANTHRAX BACILLI IN DOG SERUM.

Dog leucocytes are actively phagocytic *in vitro* for anthrax bacilli in the presence or under the influence of normal dog serum. When leucocytes—i. e., blood corpuscles or the leucocytes in pleural exudates—are washed carefully many times in NaCl solution, so as to remove all traces of serum, they no longer take up normal bacilli. The phagocytic power of washed leucocytes is restored by adding normal dog serum, but the action of the serum is not exercised directly on the leucocytes, but upon the bacilli, because bacilli that have been treated with normal serum and then washed in NaCl solution are taken up by washed leucocytes—by absorbing the

* Received for publication, January 3, 1906.

¹ *Proc. Roy. Soc.*, 1903, 72, p. 357, and 1904, 73, p. 128.

² See this number p. 156.

opsonin of the serum the bacilli become susceptible for phagocytosis. The opsonin for anthrax bacilli in dog serum is diminished greatly by heating the serum at 56° C. for 30 minutes, and as a rule,* wholly or almost wholly destroyed by heating at 60° C. for the same length of time.

The facts just stated are illustrated in Table 1, which gives the results of some of the experiments.

TABLE 1.
PHAGOCYTOSIS OF ANTHRAX BACILLI BY DOG LEUCOCYTES.

BLOOD 0.5+BACILLARY SUSPENSION 0.5	PHAGOCYTOSIS (200 LEUCOCYTES COUNTED)	
	Phagocytosis	No Phagocytosis
Defibrinated blood	192	8
Washed blood	10	190
" " + normal serum	190	10
" " + serum heated at 56° C. 30 min	60	130
" " + " " " 60° C. 30 "	30	170
" " + bacilli sensitized in normal serum	130	70
" " + " " " serum heated at 56° C. 30 min ..	44	156
" " + " " " " 60° C. 30 " ...	4	196

It is quite impossible to count the number of anthrax bacilli taken up by leucocytes; a good idea of the extent of phagocytosis is obtained, however, by counting a number of leucocytes and noting the relation between those that are and those that are not engaged in phagocytosis.

Considerable phagocytosis takes place within 15 minutes after adding bacilli to defibrinated dog blood, and gradually increases during the first hour or two. Within the leucocytes the bacilli appear to lose their power to take up stains, and disappear as shadows and irregular fragments, just as described by Metchnikoff many years ago. Often the bacilli are arranged parallel to one another in the leucocytes. Leucocytes also appear to stretch themselves along the anthrax thread, and by contracting curl up the latter within themselves. In the presence of normal serum and bacilli the leucocytes engaged in phagocytosis manifest a distinct tendency to form groups, which is not the case at all in mixtures of normal bacilli and washed leucocytes.

* Individual variations occur undoubtedly. In experiments of this kind the leucocytes used must be carefully washed free from serum.

In order to sensitize bacilli for phagocytosis a small loopful of a 24 hour agar culture is suspended in 1 c.c. of NaCl solution to which is added 1 c.c. of serum. The suspension is placed at 37° C. for 30 to 60 minutes, when the bacilli are centrifugated out, washed, and resuspended in NaCl solution. Frequently only 50 or 60 per cent of leucocytes are found engaged in phagocytosis in the case of bacilli sensitized in normal serum. This may mean that the union between opsonin and bacillus is easily destroyed and part of the opsonin lost in the washing.*

DESTRUCTION OF ANTHRAX BACILLI BY DOG LEUCOCYTES.

The question now arises whether it is possible to show by test-tube experiments to what extent, if any, phagocytosis of anthrax bacilli by dog leucocytes is associated with destruction of the bacilli. I believe that the results of the experiments recorded in Table 2 point very directly to the conclusion that phagocytosis and destruction of anthrax bacilli by dog leucocytes *in vitro* go hand in hand. In these experiments one loopful of the various suspensions of anthrax bacilli used was added to the different mixtures. By plating another loopful in each case, and counting the colonies that had developed at the end of 24 hours, the approximate total number of bacilli added was obtained, and this is the number given in the column headed "at once." Two sets of the different mixtures were made, each tube of which received one loopful of anthrax suspension; by plating the whole quantity in each tube, those of one set at the end of two or three hours, and those of the other set at the end of five hours, and then counting the colonies at the end of 24 hours, an approximately correct idea of the fate of the bacilli introduced was secured (see columns in Table 2 headed "2 hours" and "5 hours").

* It will be seen, too, from the other tables that the number of colonies is not reduced so greatly when sensitized bacilli are added to washed blood, as when normal bacilli are mixed with defibrinated blood, or with washed corpuscles and normal serum

TABLE 2.
ANTHRACIDAL ACTION OF DOG BLOOD.

MIXTURES—TOTAL QUANTITY 1 C.C.	NUMBER OF BACILLI IN TOTAL QUANTITY		
	At Once	2 Hours	5 Hours
Defibrinated blood 0.5+NaCl sol. 0.5.....	3,000	280	20
Washed blood 0.5+NaCl sol. 0.5.....	3,000	2,750	∞
Washed blood 0.5+normal serum 0.5.....	3,000	181	114
Washed blood 0.5+NaCl sol. 0.5+bacilli treated with normal serum.....	3,120	628	105
Washed blood 0.5+NaCl sol. 0.5+bacilli treated with serum heated at 56° 30 min.....	980	1,962	∞
Washed blood 0.5+NaCl sol. 0.5+bacilli treated with serum heated at 60° 30 min.....	1,540	2,036	∞
Washed blood heated at 45°C. 0.5+normal serum 0.5.....	3,500	6,475	∞
Dog serum 0.5+NaCl sol. 0.5.....	3,092	6,590	∞
Serum 0.5+NaCl sol. 0.5+bacilli treated with normal serum....	1,600	3,500	∞
Broth 0.5+NaCl sol. 0.5.....	3,092	11,550	∞
Broth 0.5+NaCl sol. 0.5+bacilli treated with normal serum.....	3,076	9,860	∞

(The sign ∞ means innumerable.)

Table 2 shows that normal anthrax bacilli grow freely in suspension of washed dog blood, in dog serum, in suspensions of washed dog corpuscles containing serum heated for 30 minutes at 56° and 60° C.; and in normal serum and washed dog blood heated at 45° for 30 minutes (there is no phagocytosis in such mixtures); further, that bacilli sensitized in the manner described in normal serum multiply in dog serum and in broth, as do also bacilli sensitized in serum heated at 56° and 60°, and then added to suspensions of washed corpuscles. Note that the corpuscular suspensions concerned in these cases are suspensions in which no or little phagocytosis takes place, as shown in Table 1. Destruction of normal bacilli takes place in defibrinated blood and in washed blood plus normal serum; destruction also takes place when bacilli sensitized in normal serum are added to suspensions of washed dog leucocytes.

When smaller numbers of bacilli (500 to 1,500), normal or sensitized, are added to defibrinated blood or to suspensions of leucocytes respectively, complete sterility may be obtained at the end of three and five hours. When the mixtures are agitated moderately in a shaking machine there is usually a greater destruction than when they stand quietly, probably because of the better opportunity for phagocytosis in the former instance.

The essential rôle of the corpuscles—leucocytes—in the destruction of anthrax bacilli by dog blood becomes very distinct in plates

made with decreasing quantities of blood and a fixed quantity of bacilli, the total quantity being made up to 1 c.c. in all cases by the addition of normal dog serum. As shown in Table 3, the de-

TABLE 3.

DIMINISHING DESTRUCTION OF ANTHRAX BACILLI WITH DECREASING QUANTITIES OF DOG CORPUSCLES (LEUCOCYTES) AND INCREASING QUANTITIES OF DOG SERUM.

QUANTITY MADE UP TO 1 C.C. BY ADDING NORMAL DOG SERUM	No. OF BACILLI IN TOTAL QUANTITY		
	At Once	3 Hours	5 Hours
Defibrinated blood 1 c.c.	1,400	0	0
" " 0.75 c.c.	"	79	560
" " 0.5 c.c.	"	89	700
" " 0.25 c.c.	"	840	3,500
" " 0.125 c.c.	"	1,750	4,650
" " 0.062 c.c.	"	1,600	10,000+

struction decreases as the amount of corpuscles decreases, in spite of the fact that there is a compensatory increase in the amount of serum. While the majority of these experiments have been made with dog blood, a sufficient number of experiments has been made with pleural exudates of dogs to indicate plainly that the same results are obtainable with the leucocytes and serum of exudates as with those of the blood, both as regards bacteriolysis (Table 4)

TABLE 4.

DIMINISHING DESTRUCTION OF ANTHRAX BACILLI WITH DECREASING QUANTITIES OF WHOLE EXUDATE FROM PLEURAL CAVITY OF DOG AND INCREASING QUANTITIES OF SERUM.

QUANTITY MADE UP TO 1 C.C. BY ADDING SERUM OF EXUDATE	No. OF BACILLI IN TOTAL QUANTITY			
	Virulent Bacilli		Avirulent Bacilli	
	At Once	5 Hours	At Once	5 Hours
Exudate 1 c.c.	3,000	43	3,200	15
" 0.75 c.c.	"	46	"	6
" 0.5 c.c.	"	17	"	27
" 0.25 c.c.	"	58	"	148
" 0.125 c.c.	"	148	"	1,050
" 0.06 c.c.	"	700	"	910
" 0.03 c.c.	"	1,750	"	1,330
Washed leucocytes from exudate 1 c.c.	"	4,700	"	3,200
Serum of exudate 1 c.c.	"	7,350	"	9,170

and phagocytosis. It is evident that in different experiments comparable results are obtainable only when the same quantity and quality of leucocytes are employed, granting that other conditions are equal.

Defibrinated blood of newborn dogs destroys anthrax bacilli to

THE OPSONIN AND AMBOCEPTOR FOR ANTHRAX BACILLI IN
DOG SERUM NOT IDENTICAL.

Now, I have found further that heating dog serum to 60° C. for 30 minutes destroys its power to render mixtures of dog serum and dog leucocytes anthracidal, but it does not interfere with its destructive action on anthrax bacilli when complemented with rabbit serum as shown in Table 5.

TABLE 5.

SUCCESSFUL COMPLEMENTATION OF DOG SERUM HEATED AT 60° C. BY NORMAL RABBIT SERUM.

[illegible]

* There is considerable individual variation in the anthracidal power of normal rabbit serum.

TABLE 6.
ACTION OF RABBIT SERUM UPON ANTHRAX BACILLI TREATED WITH DOG SERUM.

NO. OF BACILLI ADDED	NO. OF BACILLI IN TOTAL QUANTITY IN 3 HOURS		
	1 c.c. Rab. Ser.	0.05 Rab. Ser.	0.025 Rab. Ser.
Bacilli treated with normal serum—480.....	0	80	168
“ “ “ serum heated at 56°—370 .	0	33	54
“ “ “ “ 65°—656 .	0	96	46
Normal bacilli—440	4	1,000	1,066

Table 6 shows in a little different manner that the amboceptor in dog serum is not demonstrably affected by heating serum at 56° and 65° C. for 30 minutes, because bacilli treated in serum so heated are destroyed by quantities of rabbit serum so minute as to have in themselves no demonstrable lytic action upon normal bacilli. Under these circumstances it is reasonable to conclude that the anthrax opsonin and the anthrax amboceptor in dog serum are distinct bodies, and, inasmuch as dog serum, when heated at 60° C., loses its opsonin, and with it the normal power to render the bacilli destructible by washed dog corpuscles—a power possessed by normal dog serum—that the amboceptor in dog serum plays no at present demonstrable rôle in the destruction of anthrax bacilli by dog serum and corpuscles, i. e., polymorphonuclear leucocytes. And this must be so in spite of the fact that when anthrax bacilli are treated with dog serum, they probably unite with both opsonin and amboceptor.

THE ANTHRACIDAL SUBSTANCE IN DOG LEUCOCYTES.

When large quantities of dog leucocytes, obtained from aleuronat exudates, are carefully washed many times, and then extracted with sterile distilled water or allowed to undergo autolysis under toluol, a clear fluid may be secured which is destructive of anthrax bacilli. Similar leucocytic extracts have been obtained by Petterson¹ and others. The anthracidal property of the extracts that I obtained has been quite resistant to heat. There is at present no evidence to indicate that this substance, whatever it may be, plays any appreciable rôle outside of the leucocytes in the test-tube experiments recorded in this paper, because I have invariably ob-

¹ *Centralbl. f. Bakt.*, 1905, 39, p. 423.

tained a multiplication of anthrax bacilli inoculated into suspensions of washed leucocytes in NaCl solution.

SUMMARY.

Anthrax bacilli are destroyed in normal defibrinated dog blood owing to the combined action of the serum and the phagocytes.

The substance in the serum necessary for phagocytosis and destruction of the bacilli is destroyed by heat at 56° – 60° C. for 30 minutes, and it is probably not identical with the anthrax amboceptor present in dog serum.

The destructive action of dog leucocytes on anthrax bacilli in the presence of, or influenced by, normal dog serum is suspended by previously heating the leucocytes at 45° for 30 minutes, when phagocytosis no longer takes place.

Washed dog leucocytes contain a thermostable, anthracidal substance, extractible by distilled water and autolysis, but there is no evidence to show that this substance has any effect outside of the cells in the usual test-tube experiment.

Phagocytosis is an essential step in the destruction of anthrax bacilli by dog leucocytes and dog serum.

THE ANTHRACIDAL SUBSTANCE IN THE SERUM OF WHITE RATS.*

JESSIE M. HORTON.

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METCHNIKOFF,¹ more than 20 years ago, developed his theory that the property of digestion in ameboid cellular organisms is possessed also by certain cells of higher animals. The part of the phagocytes in resisting bacterial invasion he holds to be an important one, although not necessarily in every case the only means of defense; but in immunity to anthrax bacilli he gives to phagocytosis an important place, and a large part of his early experimental work was done on anthrax bacilli and spores.

When Behring,² a few years later, discovered the enormous power of the serum of the white rat to destroy anthrax bacilli, he concluded that this alone was responsible for the natural immunity which this animal possesses. There are, however, other cases that are not so easily explainable; the dog, for instance, is immune to anthrax infection, and yet its serum furnishes an excellent culture medium for the germs *in vitro*; while the rabbit, on the other hand, which is very susceptible, possesses a serum as strongly bacteriolytic in the test tube as that of the rat itself.

Behring explained the anthracidal power of the rat serum as due to alkalinity, for the following reasons:

1. Rat serum has a higher alkalinity than other sera.
2. Anthrax germs will not grow in it.
3. Through addition of acids to neutralization the serum becomes an excellent medium for anthrax.
4. Injections of oxalic acid into rats during life cause the serum to lose its anthracidal power and rob the animal of its resistance.

About a year ago Pirenne³ also came to the conclusion that the anthracidal power of rat serum, contrary to what is true of most immune sera, is due to one body which probably is an organic, basic substance. If the anthracidal power is due to the interaction of

* Received for publication December 23, 1905.

¹ *Ges. Abh.*, Leipzig, 1893.

² *Virch. Archiv*, 1884, 96, p. 502.

³ *Centralbl. f. Bakt.*, 1905, 36, p. 256.

two bodies, complement and amboceptor, the serum should possess the properties ordinarily ascribed to bacteriolytic sera in general. The amboceptor being a stable body, and highly resistant to all influences, its presence is difficult to disprove; but complement is a labile substance, easily destroyed, whose presence or absence is readily shown. Now, the following are the characteristics of the anthracidal substance in rat serum, according to Pirenne: It is thermostable, resistant to 64°C. ; its anthracidal action is lost on neutralization of the serum; it does not give rise to a specific antibody in guinea-pigs; it resists filtration, and also exposure to sun-light for 15 days; it keeps for 230 days on ice, and its action is not suspended at 0°C. For these reasons Pirenne concludes that complement in the usual sense takes no part in destruction of anthrax bacilli by the serum of the white rat.

I have repeated Pirenne's experiments, and while my results agree with his in many respects, there are important points of difference. The blood for these experiments was drawn by means of a syringe directly from the heart, under aseptic precautions, and allowed to clot against the side of a sterile glass vessel. With old rats chloroform was not given except in a few cases, as the rats could be handled by means of a towel until firmly tied down on a small board. About 4 or 5 c.c. of blood could be drawn without killing the rat, if intraperitoneal injections of salt solution were immediately given. The clear serum separating off was tested undiluted, and also diluted one part in four of normal salt solution, and as the anthracidal power was found not to be diminished by this dilution, this was then used in all subsequent tests for comparative purposes, in order to prevent coagulation on heating the serum. Heating was done in a water bath, and continued in each case for 40 minutes. In each experiment the different tubes contained the same number of drops of serum. They were inoculated with one loopful of an anthrax suspension made by rubbing up a 24 hour agar slant culture in 3 c.c. of NaCl solution, one loopful from each tube being plated at once. After four hours at 37°C. one loopful from each tube was plated again, and the colonies that developed were counted at the end of 24 hours at 37°C. An amount of broth equal to the amount of serum in each tube served as a control.

Table 1 illustrates the results obtained in these experiments.

We see that the serum of old rats (not chloroformed and not previously bled) strongly resists any temperature under 66° C., and even at this point the lytic power is only half destroyed. After heating at 68° C., the serum allows complete growth, as compared with broth. This result was obtained in numerous instances.

TABLE 1.
DESTRUCTION OF ANTHRAX BACILLI BY THE SERUM OF THE WHITE RAT.

	ONE LOOP IN EACH PLATE	
	At Once	4 Hours
Normal rat serum.....	13	0
“ “ “ heated at 58° C.....	3,144	1
“ “ “ “ “ 60.....	3,144	40
“ “ “ “ “ 62.....	3,144	60
“ “ “ “ “ 64.....	3,144	176
“ “ “ “ “ 66.....	2,800	440
Control (Broth).....	3,144	∞

In the normal unheated serum of white rats a massive destruction of bacilli appears to occur practically at once, as shown by the insignificant number of colonies that develop in the plates immediately after mixing the bacilli with the serum as compared, with the number in the plates from the control tubes of broth.

Results similar to those recorded in Table 1 were obtained by plating the entire contents of the tubes, the number of bacilli inoculated being determined by plating one loop from the anthrax suspension used for inoculating the serum. In this series also it was found that the anthracidal power of the serum is seriously impaired or completely lost only on heating at 66° and 68° C. for 40 minutes. As a general rule 0.2 c.c. of normal serum suffices to kill from 9,000 to 10,000 bacilli in four hours. Chloroform anesthesia and previous bleeding of rats appear, as was shown by Behring, to reduce somewhat the anthracidal power. All attempts at reactivating heated serum by means of serum exhausted of anthracidal substance by treatment with large quantities of anthrax bacilli were unsuccessful. In certain experiments by Hektoen and Ruediger¹ on the antilytic action of salts and other substances the serum of the white rats employed was found to have lost its bactericidal power on heating at 58° C. for 30 minutes.

¹*Jour. Infect. Dis.*, 1904, 1, p. 379.

In endeavoring to explain the discrepancy between the last-named results and those of Behring, Pirenne, and myself, the thought occurred that possibly the age of the animals might play some rôle. In order to determine the actual facts, the sera of 12 rats between the ages of three weeks and five months were tested, and it was found that the younger the rat the more thermolabile its serum, as shown in Table 2, where a summary of these experiments is given. Inquiry revealed the fact that Hektoen and Ruediger in their work used young rats.

TABLE 2.
RELATION OF AGE OF THE RAT TO THE THERMOLABILITY OF ITS SERUM.

Age of Rats	Loss of Anthracidal Power Begins on Heating at	Anthracidal Power Completely Lost on Heating at
3 weeks.....	56° C.	58° C.
1 month.....	56	58
2 months.....	56	58
3 ".....	56	60
5 ".....	58	62
Older.....	60	66

Whether the thermolability of the serum of young animals is due to the presence of complement and amboceptor early in the life of the white rat, or, as is more likely, to a smaller quantity of the same substance present in the older animals, has not been determined on account of lack of suitable animals.

Behring showed that white rat serum is more alkaline than other sera which are not destructive to anthrax; that neutralization destroys this anthracidal property; and that injections of oxalic acid during life reduce the serum to a good culture medium by reducing, so he concluded, the alkalinity. From these observations he inferred, as already pointed out, that the anthracidal action is due to alkalinity, and in this opinion he is supported by Pirenne. There are, however, certain difficulties in the way of accepting this conclusion. Thus I have found that human serum, which permits of a fairly ready growth of anthrax bacilli, is not less alkaline than rat serum. Although unquestionably anthracidal action is lost on neutralization, it is possible that the action may be due to effects other than neutralization of the reaction. Pirenne, although he agreed with Behring, found, on heating the serum to 56°—a point where the

anthracidal action was unimpaired—that the alkalinity was reduced one-quarter. I have not been able to confirm Pirenne's observations. Titrating serum, heated at 58°, 60°, and 70° C. for 40 minutes against $\frac{n}{100}$ oxalic acid, using rosolic acid as indicator, showed, in my hands, no reduction of alkalinity as compared with normal serum (Table 3). Now the fact that serum deprived of all anthracidal power by heating at 70° C. shows no loss of alkalinity suggests strongly that the anthracidal action, after all, depends on other factors—single or combined—than alkalinity, in spite of the circumstance that the serum loses its anthracidal action on neutralization by acids.

TABLE 3.
THE EFFECT OF HEAT ON ANTHRACIDAL POWER AND ALKALINITY OF RAT SERUM.

SERUM	ALKALINITY	NO. OF BACILLI IN TOTAL QUANTITY	
		At once	4 Hours
Normal serum.....	I.C.C. = 0.0014 NaOH	8,000	0
Serum heated at 70° C.....	I.C.C. = 0.0014 "	8,000	∞

Repeated injections of guinea-pigs with increasing doses of defibrinated rat blood, from 3 to 5 c.c., did not give rise to any substance that neutralized the anthracidal body in the rat serum.

While the serum of a rat five months old was deprived of its anthracidal power on exposure to direct sunlight for four days, the serum of old rats resisted this exposure longer, but not so long as in Pirenne's experiments.

It is of interest to note that the opsonic power of rat serum on anthrax bacilli, human leucocytes being used as phagocytes, closely follows its anthracidal power as regards resistance to heat, being fully destroyed only after heating at 66° C. for 30 minutes; neutralization with oxalic acid, however, has no destructive effect upon the opsonin.

CONCLUSIONS.

1. While the anthracidal substance in the serum of adult white rats, studied by Behring, Pirenne, and others, is thermostable, being destroyed by heating at 68° C. for 30 minutes, the anthracidal substances in young rats is less resistant to heat and is completely destroyed at 58° C. in rats under two months old.

2. While neutralization of rat serum with oxalic acid destroys its anthracidal power, as first pointed out by Behring, heating the serum to 68° to 70° C., at which temperature its anthracidal property is lost, does not reduce its alkalinity. Consequently the inactivation by oxalic acid would seem to depend upon more complex changes than mere reduction of alkalinity, and it also would seem likely that the anthracidal power of rat serum is not dependent on alkalinity only.

The normal serum of the white rat contains an opsonin for anthrax bacilli that is thermostable, like the bactericidal substance, but this opsonin is not affected by neutralization of the serum with oxalic acid.

THE ADJUVANT ACTION OF SERUM, EGG-ALBUMIN, AND BROTH ON TETANUS INTOXICATION.*

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(From the Pathological Laboratory of the University of Chicago.)

IN the course of other work it became necessary to assure ourselves concerning the content in tetanus antitoxin of the sera of several goats. To our surprise normal goat serum (1.0 c.c.) rather than exerting an antitoxin action, increased the toxicity of the toxin when suitable doses of the latter were used. The investigation of this phenomenon is the subject of the present paper.

TECHNIQUE AND MATERIALS.

The toxin used throughout the experiments was prepared in January, 1903, as follows: Ten liters of beef broth containing 1 per cent of glucose and having a reaction of + 1, were placed in two bottles, and inoculated with a large quantity of tetanus bacilli which had grown for 10 days in a similar broth. Washed hydrogen was then passed through the inoculated broth for an hour, and the bottles sealed and placed in the thermostat, the usual provisions being made to permit of the escape of gas from the bottles, and to prevent the access of air. After a growth of nine days the culture was passed through Pukal filters, placed in large moisture dishes, and an excess of ammonium sulphate added;† the dishes were then placed in the thermostat over night. The brownish scum which had formed by this time was skimmed off, placed between hardened filter papers, and the excess of moisture pressed out. Still more fluid and ammonium sulphate were got rid of by subjecting the precipitate to very high pressure in a pressure machine. The precipitate, now in the form of solid cakes, was dried over sulphuric acid and eventually pulverized. It is preserved over sulphuric acid in the ice-chest and in the dark.

For use a 0.2 per cent solution of the precipitate was made in 0.85 per cent sodium chloride solution, and the doses used are expressed in cubic centimeters of this solution. The original fatal

* Received for publication January 10, 1906.

† One-half more than the quantity the broth would dissolve at room temperature.

dose for white mice of about 15 grams weight was 0.000,007 c.c. per gram of mouse; death occurred in four to five days. For guinea-pigs of 250 to 300 grams, 0.000,001 c.c. per gram killed in four to five days. The toxin has undergone little or no deterioration since it was prepared.

When dealing with a substance of such high toxicity it is difficult to weigh out small quantities with desirable accuracy and at the same time observe economy of material. Our toxin solutions were usually made by dissolving 0.2 grams of the precipitate in 100 c.c. of the salt solution, but, in spite of great care in weighing, variations in the toxicity of different solutions frequently came to light. Some of these variations may have been due to an irregular distribution of the toxin in the precipitate. A new toxin solution was prepared for each experiment.

White mice were used as the test animals throughout the work, and the dosage of toxin was based on the gram-weight of the animals—i. e., so much toxin per gram. It was found, however, that this method could be used safely only when the various mice of a given experiment were approximately of the same weight, since, for example, 0.000,007 c.c. of toxin per gram is not equally toxic for a 20 gram and a 10 gram mouse. Susceptibility is not directly proportional to weight.

The injections were made into the loose subcutaneous tissue of the back.

We soon learned that the ability of normal serum to increase the toxicity of the toxin disclosed itself only when suitable doses of the toxin were used. If a dose of toxin which killed all controls in 48 to 72 hours was used, the increased toxicity caused by the addition of serum did not become manifest. Also if the dose was so small that very little tetanus resulted in any of the animals, the effect of the serum was not always a decisive one. The clearest results were obtained when a dose of toxin was used which either caused moderate tetanus in the controls with eventual recovery, or which caused their death in from eight to twelve days.

Because of the possibility of variations in the toxicity of toxin solutions, one could not feel sure that a single dose selected for an experiment would be the optimum dose for the manifestation of the phenomenon. Hence it was decided to use a number of mice for

each experiment, and to vary the dosage in such a way that an optimum dose would be administered to two or more of the animals. It was necessary to use an abundant number of controls.

The sera of a number of normal goats were used, also of normal rabbits, and for specific purposes which will be explained later, the influence of broth and of egg-albumin was tested. Customarily 1 c.c. of the serum (or broth, or egg-albumin) was mixed with a dilution of the toxin of which 0.5 c.c. contained the desired dose. The mixture was allowed to stand for varying lengths of time and the total quantity (1.5 c.c.) then injected into the subcutaneous space in the back of the white mouse by means of a Luer or Roux syringe. After the mixture had been injected an additional 0.5 c.c. of salt solution was drawn into the syringe, and, after rinsing, was injected. In order to prevent any escape of fluid after its injection, the needle puncture was clamped for 15 to 30 minutes, the clamp being applied before the needle was withdrawn.

In all experiments daily observations were made of the degree of tetanus present, as indicated by the amount of deformity and the general appearance of the animals. The degree of tetanus is expressed in the tables by figures; thus 0 = no tetanus, 1 = perceptible rigidity; 2 = distinct but not pronounced; 3 = marked rigidity; 4 = severe; 5 = very severe to moribund; † = death. The observations of one of us served as a check on those of the other.

EXPERIMENTS.

Tables 1, 2, and 3, which follow, are typical experiments showing the influence of normal goat and rabbit sera.

TABLE 1.

THE INFLUENCE OF NORMAL GOAT SERUM ON TETANUS INTOXICATION.

Normal serum, Goat 1.—Serum three days old, unheated, preserved in the ice-chest. † Serum and toxin mixed and allowed to stand 30 minutes at 37 C.° before injection.

MOUSE	WT. IN GRAMS	TOXIN, PER GRAM	C.C. OF SERUM	RESULT BY DAYS											
				1	2	3	4	5	6	7	8	9	10	11	15
1.....	14	0.000.001	1.0	0	1	2	1	1	1	1	1	1	1	1	0
2.....	14	0.000.001	0.0	0	0	1	1	1	1	1	1	0	0	0	0
3.....	16	0.000.003	1.0	0	0	2	2	2	2	1	0	1	1	1	0
4.....	10	0.000.003	0.0	0	1	2	2	1	1	1	1	0	0	0	0
5.....	14	0.000.006	1.0	0	2	3	4	4	5	†					
6.....	14	0.000.006	0.0	0	1	1	1	1	1	1	0	0	0	0	0
7.....	14	0.000.008	1.0	0	1	4	†								
8.....	14	0.000.008	0.0	0	2	2	3	3	4	3	3	3	2	1	0
9.....	13	0.000.01	1.0	0	3	5	†								
10.....	13	0.000.01	0.0	0	1	2	3	3	3	1	1	1	1	0	0

TABLE 2.

Same as Table 1. except that the serum of Goat II was used.

MOUSE	WT. IN GRAMS	TOXIN PER GRAM	C.C. OF SERUM	RESULT BY DAYS												
				1	2	3	4	5	6	7	8	9	10	11	12	13
1.....	14	0.000.001	1.0	0	1	1	1	1	1	1	1	0	0	0	0	0
2.....	14	0.000.001	0.0	0	0	1	1	1	1	1	1	0	0	0	0	0
3.....	15	0.000.003	1.0	0	2	3	4	4	4	4	5	+				
4.....	15	0.000.003	0.0	0	1	1	1	2	3	3	3	2	2	2	1	0
5.....	14	0.000.006	1.0	0	1	3	3	4	4	4	4	4	+			
6.....	14	0.000.006	0.0	0	1	3	3	2	2	1	1	1	2	2	1	0
7.....	14	0.000.008	1.0	0	2	3	5	+								
8.....	14	0.000.008	0.0	0	1	3	3	4	5	+						
9.....	14	0.000.01	1.0	0	3	5	+									
10.....	14	0.000.01	0.0	0	2	2	3	3	3	2	2	2	(lost)			

TABLE 3

THE INFLUENCE OF NORMAL RABBIT SERUM ON TETANUS INTOXICATION.

Serum, unheated, two days old. Mixtures stood for one and one-half hours at room temperature before injection.

MOUSE	WT. IN GRAMS	TOXIN PER GRAM	C.C. OF SERUM	RESULT BY DAYS						
				1	2	3	4	5	6	7
1.....	13	0.000.003	1.0	0	2	3	3	5	†	
2.....	11	0.000.003	0.0	0	2	3	3	4	5	†
3.....	16	0.000.006	1.0	0	3	4	†			
4.....	15	0.000.006	0.0	0	3	4	5	5	†	
5.....	19	0.000.008	1.0	0	4	†				
6.....	16	0.000.008	0.0	0	2	2	4	†		

Tables 1, 2, and 3 require little or no comment. Particularly in Tables 1 and 2, there can be no question concerning the ability of the serum to increase the intensity of the tetanus intoxication from which death occurred. Table 3 is somewhat less decisive, but here also all animals receiving serum died from one to three days in advance of the controls.

TABLE 4.

TO DETERMINE THE MINIMUM QUANTITY OF GOAT SERUM WHICH INTENSIFIES THE INTOXICATION.

Fresh serum, 18 hours old, from a normal Angora goat.

MOUSE; WT. 10 GRAMS	TOXIN PER GRAM	C.C. OF SERUM	RESULT BY DAYS					
			1	2	3	4	5	6
1.....	0.000.008	0.0	0	0	2	4	5	†
2.....	0.000.008	0.0	0	2	3	4	5	†
3.....	0.000.008	0.01	0	1	3	†		
4.....	0.000.008	0.05	0	2	3	†		
5.....	0.000.008	0.10	0	2	†			
6.....	0.000.008	0.40	0	1	3	5	†	
7.....	0.000.008	0.70	0	1	1	3	5	†
8.....	0.000.008	1.00	0	1	1	3	†	

In accordance with Table 4, 0.1 c.c. of serum is a more powerful adjuvant than 1 c.c., the latter being the dose used uniformly. This point was determined only after the major part of the experiments had been completed. Its explanation is by no means clear. The serum may contain a minute amount of antitoxin, which declares itself when larger quantities are used.

Experiments of which Table 5 is an example, were performed to determine whether the effect of the serum could be attributed to its toxic action on the mice. If such a toxic action were present, the early death of the animals might be referred to a summation of the intoxication by the serum and that by the toxin. We looked on the subsequent weight of the animals as a clue to the presence or absence of serum intoxication.

TABLE 5.

TO DETERMINE THE TOXICITY OR NON-TOXICITY OF NORMAL GOAT SERUM FOR WHITE MICE.
Serum three days old, unheated.

MOUSE	WT. IN GRAMS	C.C. OF SERUM	WEIGHT BY DAYS				
			1	2	4	5	6
1	17	1.0	18	19.5	18	18	18
2	14	1.5	15	17.0	16	15	15
3	17	2.0	18	20.0	20	18	18

There was no loss of weight, but even a gain on the second day, which may have been due to the drinking of more water. Hence we conclude that the phenomenon cannot be explained by any manifest toxicity of the serum for the mice.

Other experiments were performed with the hope that some light might be thrown on the nature of the phenomenon. It occurred to us that tetanus toxin might be an amboceptor, and that the normal serum of the goat might contain suitable complement which, when injected, would increase the amount of suitable complement in the body of the mouse, and thus activate the toxin more quickly and more completely. To throw possible light on this point the comparative influence of fresh, old, and heated sera was determined (Table 6).

The experiment shows distinctly that the effect of the serum does not depend on an activation of hypothetical tetanus amboceptors by ordinary thermolabile complement. The heated and old

TABLE 6.

THE COMPARATIVE INFLUENCE OF FRESH, HEATED, AND OLD SERA ON TETANUS INTOXICATION.

The sera were from Goat IV (female); the "heated" serum had been placed at 56° C. for 30 minutes and was freshly drawn; the "old" serum had been drawn 11 days previously and kept at about 10° C.

MOUSE	WT. IN GRAMS	TOXIN PER GRAM	I C.C. OF SERUM	RESULT BY DAYS														
				1	2	3	4	5	6	7	8	9	10	11	14	19		
1.....	18	0.000,006	Fresh	0	1	2	3	4	4	..	5	†						
2.....	18	0.000,006	Heated	0	1	2	3	3	3	..	5	†						
3.....	18	0.000,006	Old	0	1	2	3	4	†									
4.....	18	0.000,006	None	0	1	2	3	3	3	..	4	3	..	2	3	0		
5.....	18	0.000,008	Fresh	0	0	3	3	4	4	†								
6.....	18	0.000,008	Heated	0	1	3	4	†										
7.....	18	0.000,008	Old	0	1	3	†											
8.....	18	0.000,008	None	0	1	2	3	3	3	..	4	3	..	2	1	0		

sera were rather more efficient than the fresh serum. Serum heated even to the coagulating point does not lose its adjuvant property (Table 7).

TABLE 7.

HEAT RESISTANCE OF THE ADJUVANT SUBSTANCE.

MOUSE; WT. 12 GRAMS	TOXIN PER GRAM	I C.C. GOAT SERUM HEATED TO —° C. FOR 30 MIN.	RESULT BY DAYS							
			1	2	3	4	5	6	7	8
1.....	0.000,007	67.5-68*	0	1	3	†				
2.....	0.000,007	67.5-68	0	3	†					
3.....	0.000,007	64.5-65	0	1	3	†				
4.....	0.000,007	64.5-65	0	1	4	†				
5.....	0.000,007	59.5-60	0	1	3	†				
6.....	0.000,007	59.5-60	0	3	†					
7.....	0.000,007	Unheated	0	1	4	†				
8.....	0.000,007	Unheated	0	1	4	†				
9.....	0.000,007	No serum	0	0	1	2	2	2	1	0
10.....	0.000,007	No serum	0	0	1	1	1	1	0	

*The serum at 67.5°-68° was gelatinous. Temperatures higher than 68° C. were not tried.

Other experiments seem to show that the influence of the serum does not depend on any action on the toxin itself. It is not necessary to mix the serum and toxin before injection in order to get the effect of the former. The two may be injected in different parts of the body, or the serum may be injected shortly in advance of the toxin. It is well known, on the other hand, that a substance which acts directly on the toxin (tetanus antitoxin) has a stronger effect when the two are mixed before injection. In order to get the maximum effect of the serum, however, it is necessary that the two substances be injected in fairly close sequence, although they may be placed in different parts of the body (Table 8).

TABLE 8.

THE EFFECT OF THE SERUM WHEN INJECTED AT DIFFERENT PERIODS IN ADVANCE OF THE TOXIN.

I C.C. SERUM HOURS IN ADVANCE OF TOXIN	MICE 10 GRAMS WEIGHT	TOXIN PER GRAM	RESULT BY DAYS											
			1	2	3	4	5	6	7	8	9	10	11	12
46 hours	No. 1	0.000,008	0	2	2	3	4	4	3	3	..	4	..	†
21 "	" 2	0.000,008	0	3	4	4	4	5	5	5		†		
10 "	" 3	0.000,008	0	3	3	4	5	†						
5 "	" 4	0.000,008	0	3	4	4	4	5	†					
Simultaneous	" 5	0.000,008	0	3	4	†								
No serum	" 6	0.000,008	0	2	2	3	3	4	4	4	..	5	..	†

The results of this experiment give us the impression that the action of the serum depends on some temporary influence which it exerts on the tissues of the animal, whereby the latter is made more susceptible to the toxin. After the injection of the serum its influence gradually becomes less, and is not demonstrable after the lapse of about 46 hours.

It is equally important from the standpoint of interpretation to know whether the serum intensifies intoxication when given subsequent to the injection of the toxin. The result of a single experiment indicates that the serum does not hasten the death of the animals when it is given subsequent to the binding of the toxin by the tissues. The controls which received serum and toxin simultaneously died in five days, while animals in which the injection of the serum was given from one to fifty hours later than that of the toxin either recovered or died in from six to nine days.

We had come to believe at this time that there was nothing of a specific nature in the phenomenon, and that many other proteid-containing substances might have a similar influence. We found this to be strikingly true in the cases of egg-albumin and broth (Tables 9 and 10.)

We have before us then the following facts upon which we may attempt to base conclusions:

1. The normal sera of the goat and rabbit intensify and hasten the course of tetanus in white mice, when suitable doses of our precipitated toxin are used (Tables 1, 2, and 3).

2. It is immaterial whether the serum is fresh, old, or heated to the coagulating point (Tables 6 and 7).

3. The effect is most pronounced when the serum is injected

TABLE 9.

THE INFLUENCE OF EGG-ALBUMIN ON TETANUS INTOXICATION.
One per cent of egg-albumin in 0.85 per cent NaCl solution.

MOUSE	WT. IN GMS.	TOXIN PER GRAM	C.C. OF ALBUMIN	RESULT BY DAYS								
				1	2	3	4	5	6	7	8	9
1.....	15	0.000,005	0.5	0	1	2	3	4	†			
2.....	15	0.000,005	0.0	0	0	0	0	†	1	0	0	0
3.....	15	0.000,006	0.5	0	1	2	5	†				
4.....	15	0.000,006	0.0	0	0	0	1	1	1	1	1	0
5.....	15	0.000,007	0.5	0	1	3	†					
6.....	15	0.000,007	0.0	0	0	1	1	1	1	1	0	0

TABLE 10.

THE INFLUENCE OF BROTH ON TETANUS INTOXICATION.

MOUSE	WT. IN GMS.	TOXIN PER GRAM	C.C. OF BROTH	RESULT BY DAYS										
				1	2	3	4	5	6	7	8	9	10	11, 12, 13
1.....	15	0.000,006	1	0	0	3	3	4	†					Recovery
2.....	15	0.000,006	0	0	0	1	2	3	3	4	4	4	3	
3.....	18	0.000,007	1	0	1	3	†							
4.....	18	0.000,007	0	0	1	2	3	4	5	†				
5.....	15	0.000,008	1	0	1	†								
6.....	15	0.000,008	0	0	1	2	4	†						

simultaneously with, or shortly preceding, the injection of the toxin. If the serum is given 46 hours in advance of the toxin it is in some way disposed of so that it no longer intensifies the intoxication. It is not necessary that the serum be mixed with the toxin, nor injected into the same part of the body (Table 8).

4. A small quantity of serum (0.1 c.c.) seems to have a more pronounced influence than a larger quantity (1 c.c.) (Table 6).

5. Normal goat serum in a quantity of 2 c.c. produces no perceptible deleterious effect on the mouse (Table 5).

6. One-half c.c. of a 1 per cent solution of egg-albumin in physiologic salt solution; and also 1 c.c. of broth have an influence like that of serum.

The possibility which we at one time took under consideration that tetanus toxin might be an amboceptor, and that the serum increases toxicity because it provides an additional quantity of complement cannot be entertained: first, because old and heated serum, in which there is no complement (thermolabile), produce the phenomenon; and, second, because egg-albumin or broth may be sub-

stituted for the serum with the same result. We cannot accuse the broth, in particular, of containing complement.

Inasmuch as we have not been able to refer the phenomenon to any influence which the adjuvant substances may exert on the toxin, we have been obliged to assume that it is due to some effect on the tissues of the mice. Although the serum exerted no perceptible toxic action on the mice, which if it had occurred might have lessened resistance in some manner, we were bound to consider as a possibility that the serum, albumin, and broth might exert some particular influence on the nervous tissue whereby it either absorbed more toxin or was made less resistant to the toxin which it bound. In spite of this possibility, however, we have been unable to conceive of any manner in which these substances could produce such an effect on the nervous tissue. If the serum, etc., were to some extent bound as indifferent food substances by the nervous cells, we believe the latter might be preoccupied, so to say, in digesting them; but since such a process would engage the cells in a general way we believe their affinity for some second substance, such as tetanus toxin, would be decreased rather than increased during this period. Concerning the alternative possibility that the serum, etc., may injure the nervous cells so that they are less resistant to the toxin, we have only the argument that no injury detectable by gross means was produced.

We find what appears as a more reasonable explanation of the phenomenon in an influence which the serum, etc., may exert on the remaining tissues of the body other than the nervous tissue. We learn from certain investigations by Metchnikoff,¹ and by Roux and Borrel,² that other tissues than the nervous are able to bind tetanus toxin in some instances. The rabbit and also the chicken are much more susceptible to tetanus toxin when it is injected into the nervous tissue than into the subcutaneous tissue; in the latter instance the toxin comes in contact with various tissues some of which bind a certain amount. Metchnikoff found that the liver in some of the invertebrates absorbs a great deal of tetanus toxin. We are not aware of any experiments bearing on the antitoxic powers of the organs of the white mouse, nor have we performed

¹ *L'Immunité*, Paris, 1901, p. 343.

² *Ann. de l'Inst. Pasteur*, 1898, 12, p. 229.

such experiments. Wasserman and Takaki found that small quantities of the liver, kidney, spleen, and bone-marrow of the guinea-pig exerted no antitoxic action, a condition which corresponds well with the exquisite susceptibility of this animal to tetanus. In comparing the susceptibility of the rabbit with that of the guinea-pig, we learn, first, that, gram for gram, the rabbit is about a thousand times more resistant than the guinea-pig (Knorr, cited by Dieudonné¹); and second, from Roux and Borrel, that a large part of this resistance resides in other than nervous tissues. The *tetanus sine tetano* of Dönitz suggested to him that non-nervous organs in the rabbit bind tetanus toxin.² Such animals after receiving a dose of toxin which causes little or no tetanus gradually become emaciated and die.

We cannot of course hold, without definite experimental proof, that the same conditions exist in the mouse. We know, however, that the mouse, gram for gram, is from six to ten times more resistant to tetanus toxin than the guinea-pig (Knorr³); in our own experiments it is seven times more resistant, and it is quite possible that some of this resistance depends on the ability of tissues other than the nervous to bind a certain amount of the toxin.

If this condition exists, and if in some way union of the toxin with other tissues could be prevented, for example, with the liver, connective tissues, or lymphoid organs, just so much more toxin would be available for the more susceptible nervous tissue.

We conceive that such a result may be caused by the serum, etc., in one of two ways: First, certain receptors or substances in the serum, egg-albumin, and broth, may possess cytophilous haptophores, identical with that of tetanus toxin, and by uniting with the tetanophile receptors of indifferent organs (liver, etc.) may thereby render impossible the binding of the toxin; this extra toxin would then be available for the nervous tissue, which presumably has a higher affinity than other tissues for the toxin. Such a process would consist of a preoccupation of tetanophile receptors by heterogeneous substances.

Second, the cells of indifferent organs (liver, etc.) may bind the serum, etc., as they would bind food substances, after which their

¹ *Immunität, Schutzimpfung, etc.*, Leipzig, 1903, p. 13.

Cited by Dieudonné, *loc. cit.*

² *Deutsche med. Wchnschr.*, 1897, 23, p. 428.

activities (ferments) may be directed toward the digestion or oxidation of the new substances, and being thus engaged, the affinities of their receptors for other substances (toxin) may be decreased. This condition would also render more toxin available for the nervous tissue. Such a process would consist of a non-specific engagement of the activities of the cells, without a direct occupation of tetanophile receptors.

As an example of such a process we may mention the well-known experiments of Besredka,¹ in which granules of carmin injected into the peritoneal cavity decreased the ability of the leucocytes to take up granules of arsenic, with the result that the animals died the more readily of arsenic intoxication.

Von Dungern also observed a clear example of the ability of one substance to interfere with the absorption of a second.² By injecting the plasm of *Octopus vulgaris* or egg-albumin into the circulation of a rabbit the ability of the animal to absorb a subsequent injection of the plasm of *Maja squinado* (spider crab) was largely or entirely inhibited. Von Dungern's explanation of this phenomenon is somewhat incomplete, but we understand it to be the following: In reference to the particular substances injected, the cells of the rabbit have two types of receptors. One type is that which is able to take up various materials as food substances and is not a specific receptor. It takes up egg-albumin, the plasm of the spider crab and of the octopus, and doubtless many other albumins. It is not concerned in the formation of antibodies. The other type is specific, can take up only one particular substance, and when it proliferates, is cast into the circulation as an antibody.

Now when the plasm of the octopus or egg-albumin was injected into the rabbit, the first type of receptor, the non-specific type, was occupied largely or completely, and hence was not able to fix the plasm of the crab which was injected two and one-half hours later. Only the second type, the specific receptor, remained to fix the plasm of the crab. As a consequence the last-named substance disappeared from the circulation much more slowly than when it was injected into an untreated rabbit.

¹ *Ann. de l'Inst. Pasteur*, 1899, 12, pp. 49 and 200.

² *Die Antikörper*, Jena, 1903, p. 97.

We interpret the phenomenon which we have described in a somewhat similar way, although it is necessary for us to introduce the consideration that, the tissues of the mouse having united with the serum, egg-albumin, or broth, they thereby lose in their affinity for tetanus toxin. After the foreign substances have been disposed of by the cells, the latter again reach a state of equilibrium, and their tetanophile receptors regain their former affinity for the toxin (Table 8).

It is not possible to assume, on the basis of our experiments, that an antitoxic or bactericidal serum can in any way intensify the corresponding disease, provided the serum is sufficiently rich in antibodies. In diphtheria, for example, the quantity of antitoxin introduced is able to neutralize all the toxin which happens to be in the body, hence there is no possibility of intensifying the diphtheritic intoxication.

FURTHER STUDIES ON VIRULENT PSEUDODIPHTHERIA BACILLI.*

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INTRODUCTION.

IN the *Journal of Infectious Diseases*, 1904, 1, p. 690, a variety of virulent pseudodiphtheria bacilli was described, 15 strains of which had been isolated from the throats of persons suffering from various diseases, including scarlet fever, measles and diphtheria. The 15 strains differed somewhat in minor characteristics, but all were virulent, and the pathological effect upon guinea-pigs was not neutralized by diphtheria antitoxin, but was neutralized by the serum of a rabbit immunized against one of the group. Most of the strains corresponded culturally and morphologically to the usual descriptions of pseudodiphtheria bacilli, but some resembled closely *B. diphtheriae*, from which they could be distinguished with certainty only through animal experiments. The term "virulent pseudodiphtheria bacilli" was applied to all these strains on the theory that they represented a distinct group within the large, loosely formed, class of organisms comprehended under the term "pseudodiphtheria bacilli."

It was found that the guinea-pigs injected with one of these organisms (the lethal dose being $\frac{1}{2}$ to 1 per cent of the body weight) died usually within the following 24 hours, and that the changes found at autopsy differed from those found after the injection of *B. diphtheriae* in several respects; namely, absence of subcutaneous edema, absence of characteristic changes in the adrenals, more marked congestion of the liver and kidneys, presence of bacteria in large numbers in all organs and fluids. No soluble toxin was formed by the organisms, and the filtered cultures were entirely free from toxicity.†

*Received for publication January 5, 1906.

† After the publication of this work Dr. William Hallock Park called our attention to an article published in the *Proceedings of the New York Pathological Society* in 1898, by Louise Dudley Davis working in Dr. Park's laboratory, in which were described 39 strains of diphtheria-like organisms isolated from cases of scarlatinal otitis media, 12 of which proved pathogenic for guinea-pigs, producing in them general bacteriemia. Diphtheria antitoxin exerted no protection against these organisms. In all probability the bacilli described later by Dr. Ruediger (*Trans. Chicago Path. Soc.*, 1903, 6, p. 45) and many of those studied by us, are closely related to those isolated by Miss Davis.

The present article endeavors to take up the question as to the proper classification of these bacilli, and as to the nature of the protective serum.

CLASSIFICATION OF THE SO-CALLED VIRULENT PSEUDODIPHTHERIA BACILLI.

It may be taken for granted that any question which is discussed and settled each year for many successive years in different ways by different observers, is still an unsettled question, presenting unusual difficulties. Such is the question of the differentiation between the Klebs-Loeffler bacillus and the pseudodiphtheria bacillus. Hardly a year passes without bringing a number of articles on the subject of similarities and dissimilarities of the two organisms, and on the proper method of distinguishing them. Over and over again a sure and satisfactory method is propounded, only to be rejected later as unsatisfactory in the hands of other observers. It is unnecessary to go into detail as to the varying views on this subject; for that, reference may be made to the article already mentioned. Sufficient to say that the scientific world is still divided into: first, the strict unitarians, who repudiate the term pseudodiphtheria bacillus altogether, and insist that there is but one organism; second, those that hold that the Klebs-Loeffler bacillus is one organism and the Hoffmann-Wellenhof bacillus quite distinct from it; third, those who look upon the latter as not a single bacillus, but a group of allied bacilli; fourth, those who insert a third group of "diphtheria-like" bacilli between *B. diphtheriae* and *B. pseudodiphtheriticus*; and, finally, those who hold that the term *B. diphtheriae* as well as *B. pseudodiphtheriticus* covers a group of organisms with several subdivisions.

In the face of this difference of opinion on the part of the most eminent authorities—each laboratory practically having its own special theory—it is no easy task to attempt to classify any doubtful strain of diphtheria-like bacilli according to the ordinary tests; i. e., on the basis of morphology, chemical activity, cultural characteristics, or virulence for guinea-pigs. By this we do not mean to assert that the typical pseudodiphtheria bacillus cannot be readily distinguished from the typical diphtheria bacillus. Such strains present, as a usual thing, little difficulty. It is the atypical strains which cause

the trouble—the short, solid forms of *B. diphtheriae*, growing abundantly and occasionally forming pigment; the diphtheria-like strains of pseudodiphtheria, growing scantily on agar, forming acid in broth, and resembling morphologically the Klebs-Loeffler bacillus. Occasionally, as in Nos. 5 and 11 of our strains, there is nothing in morphology or cultural characteristics which distinguishes these bacilli from typical diphtheria bacilli. In such cases, where the ordinary tests fail, a rational method lies at hand in the use of specific sera.

Spronck made use of such a method to show that the xerosis and pseudodiphtheria bacilli are absolutely distinct from *B. diphtheriae*, since the slightly virulent strains of the first two call forth the same symptoms in guinea-pigs protected by diphtheria antitoxin as in those not so protected, showing that their virulence is not due to the production of the specific toxin of diphtheria. Neisser and Glücksmann proved that animals treated with repeated injections of pseudodiphtheria bacilli are not thereby rendered immune to *B. diphtheriae*, and Petrie showed that filtered cultures of pseudodiphtheria bacilli are not capable of producing in horses immunity to the toxin of diphtheria. The objection to Spronck's method is that it can be applied only to organisms which possess some degree of virulence, and therefore not to the avirulent diphtheria bacilli, or to the great majority of pseudodiphtheria bacilli. If, however, it were possible to find a serum bactericidal to certain pseudodiphtheria bacilli, and not to *B. diphtheriae*, such a serum could be used as a means of differentiating the two classes of organisms in those cases in which morphological, chemical, and cultural tests have proved unsatisfactory. The bacilli which responded to the bactericidal serum would then be grouped in a large class, including varieties which might differ in virulence and, to a certain extent, in staining properties, acid production, etc.

In the article already referred to, details were given as to the animal experiments performed with 15 strains of virulent pseudodiphtheria bacilli. Guinea-pigs injected with broth cultures of these strains died from general bacteriemia, unless they were protected by the serum of a rabbit which had been immunized against one member of this group. The organisms were, therefore, shown to belong

to a class distinct from *B. diphtheriae*, for the protective serum was found to have no power to protect guinea-pigs against injections of *B. diphtheriae*. Normal rabbit serum did not protect against a lethal dose of these bacilli.

Animal experiments of this kind are applicable only to virulent organisms, and in order to discover whether there were avirulent members of this same group of bacilli, it was necessary to substitute experiments in the test tube. The protective serum proved bactericidal *in vitro* as well as *in vivo*, and the test-tube experiments showed that normal rabbit serum also, although quite without protective action *in vivo*, had a transient but often marked bactericidal effect in the test tube. In the course of these experiments we tested the action of normal and immune rabbit serum on 71 strains of diphtheria and pseudodiphtheria bacilli. These included 13 of the original 15* strains; and in addition 10 strains of "diphtheria-like" bacilli kindly furnished by Dr. Anna Williams from the laboratory of the New York City Department of Health; seven strains of Westbrook's Type D 2 sent by Dr. O. McDaniel from the laboratory of the Minnesota State Department of Health; and 41 strains of diphtheria and pseudodiphtheria bacilli which were isolated by various workers in this laboratory in the course of routine examinations of throat, nosé, ear, and urine.

Out of all this material only five strains were added to the 13; that is, only five were shown to have the same receptors as the bacilli already described in the former article. Five more were found to be closely related to them, as they were killed by rabbit serum; but in the case of these last, the immune rabbit serum was no more strongly bactericidal than normal rabbit serum, while in the case of the five above mentioned the immune serum was much more strongly bactericidal than was normal serum. The other 48 strains were unaffected by the presence of rabbit serum, except when it actually appeared to favor their growth.

These 18 strains, then, form a distinct group, distinct from *B. diphtheriae* on the one hand, and from other pseudodiphtheria bacilli on the other. We have been unable thus far to find any single cul-

*No. 8 was overlooked and the cultures allowed to dry up. No. 10, which had apparently responded positively to the protective serum in the guinea-pig experiments, failed to respond in the test tube.

tural, morphological, or chemical characteristic which will distinguish some of them from the ordinary pseudodiphtheria bacilli, or others of them from diphtheria bacilli; the serum test seems the only positive one. We tested all our strains with reference to their behavior toward Gram's stain, their ability to hemolyze rabbit corpuscles, and their ability to ferment the dextrin serum-water medium of Hiss; but we can only say that our organisms resemble pseudodiphtheria bacilli rather more closely than diphtheria bacilli in these respects.

As to Gram's stain, it is notoriously unsatisfactory as applied to *B. diphtheriae*, for these bacilli are easily decolorized if left long enough in alcohol, and the method therefore gives varying results in the hands of different workers. Indeed, there are textbooks on bacteriology in which *B. diphtheriae* is placed among the Gram-negative organisms.¹ This want of agreement as to the staining properties of *B. diphtheriae* is usually explained on the grounds of faulty technique; those who obtained negative results are said to have used old cultures or to have kept their smears too long in alcohol.² Still there are not lacking reports of typical, virulent Klebs-Loeffler bacilli, which, in spite of most careful and approved staining methods, are decolorized by Gram (Zupnik,³ Schick, and Ersetting⁴). It is admittedly a test which depends very largely on the personal equation, and is therefore of limited value. We have found few statements in the literature as to the behavior of pseudodiphtheria bacilli to Gram's stain, yet they are undoubtedly considered in general as Gram-positive bacilli, and we must admit that our results are unusual in this regard. Of the 18 strains which are killed by our immune serum, 11 are Gram-negative,* and seven Gram-positive. All of the five strains which respond equally to normal and immune rabbit serum are Gram-positive, and of the 48 strains which were unaffected by rabbit serum, all but four are Gram-positive. It may be argued that the Gram-negative bacilli should be relegated to another group, and that the term "pseudodiphtheria bacillus" cannot be applied to Gram-negative organisms. In reply to this, we can only say that we consider the characteristics which these strains

¹PLAUT, *Deutsche med. Wchnschr.*, 1894, 20, p. 920.

²CZAPLEWSKI, *Hyg. Rundschau*, 1896, 6, p. 1029.

³*Prag. med. Wchnschr.*, 1902, 271, p. 361.

⁴*Wien. klin. Wchnschr.*, 1903, 16, p. 993.

*Plaut's method was followed in staining our cultures (see reference above).

have in common with the Gram-positive pseudodiphtheria bacilli more important and significant than their failure to respond to a stain, which is generally looked upon as only a partially satisfactory test for these bacilli. The bacilli which are Gram-negative may be used to produce in rabbits a serum which both agglutinates and is bactericidal to the Gram-positive bacilli of this group.

It has been shown by several observers that the power to hemolyze rabbit's corpuscles is possessed by the great majority of strains of *B. diphtheriae*. According to Schwoner, 70 per cent is about the proportion of hemolyzing strains. The pseudodiphtheria bacillus is said by the same observer to be non-hemolyzing. All of our typical Klebs-Loeffler bacilli proved to be hemolyzers, while the typical pseudodiphtheria bacilli failed to hemolyze. As one would expect, some of the "diphtheria-like" bacilli (five in all) hemolyzed, but the majority (16) did not. This is, however, a test which must be used early, for many strains lose their hemolytic power after long growth on artificial media. Only two of the 18 organisms of our group hemolyzed rabbit corpuscles.

According to Knapp,¹ it should be possible to distinguish *B. diphtheriae*, *B. xerosis*, and *B. pseudodiphtheriticus* by fermentation tests, using the serum-water medium of Hiss with the addition of certain sugars. The first should ferment all but saccharose, the second all but dextrin, and the third none at all. We followed Knapp's procedure with much care, but were unable to confirm his results in any respect except as regards dextrin, and then only partially. The typical *B. diphtheriae* caused coagulation, or acid formation, or both, in the dextrin tubes in every case; the typical pseudodiphtheria bacillus did not. But in the case of the atypical forms (including Westbrook's Type D 2), the results were various. We had five negative results as against 13 positive results, so that the test fails in the very cases in which it should be of use. In this case, too, the 18 bacilli of our group resemble pseudodiphtheria, for only two ferment dextrin. We have, thus far, found no organism which both ferments dextrin and hemolyzes rabbit's corpuscles, except typical *B. diphtheriae*.*

¹Jour. Med. Res., 1904, 12, p. 475.

* Since writing the above we have isolated from the throat of a case of diphtheria, along with a typical *B. diphtheriae*, an organism which grows like the less abundant forms of pseudodiphtheria bacillus is not virulent, is killed by our immune serum, and which ferments dextrin and hemolyzes rabbit's corpuscles.

The term "pseudodiphtheria bacillus" is loose and unsatisfactory. It may be that organisms represented by our 18 strains should be altogether separated from the pseudodiphtheria group and relegated to a class by themselves. Still, these bacilli correspond to the descriptions of pseudodiphtheria bacilli in the literature, having no distinctive feature except their virulence and their behavior to rabbit serum. All of this group which we have isolated, except two strains, were virulent for guinea-pigs immediately after isolation, and some of them are still so even after a lapse of over two years. Ordinarily the pseudodiphtheria bacillus is non-virulent, but virulent forms have often been reported. As to the effect of rabbit serum, it remains to be seen whether there is not a fair proportion of pseudodiphtheria bacilli which will be found sensitive to the action of normal rabbit serum. At the present stage of our work we are inclined to believe that the term "virulent pseudodiphtheria bacilli" is the most convenient and accurate designation for these organisms.

STUDY OF THE NATURE OF THE IMMUNE SERUM.

The bacteriolysin.—In immunizing our rabbits successive doses of broth cultures of *B. No. 1* (Ruediger) were injected under the skin at intervals of seven days, beginning with 1 c.c. of a 24 hour broth culture, and increasing by 1 c.c. each time until 7 to 12 doses had been given. The serum is decidedly bactericidal after the fourth dose, and may then be used to protect guinea-pigs, but the bactericidal substance increases if the doses are repeated up to about 10 or 12 doses. The individual rabbits vary a good deal in respect to immunization, some yielding a stronger serum after the seventh dose than others after the twelfth. There is nothing to be gained by increasing the amount given at each dose. After thorough immunization the serum of a rabbit retains its full bactericidal power for only about six weeks, after which it begins to lose its effect.

In making bactericidal tests the following method was used: For each strain five tubes were prepared, the first containing 1 c.c. of plain broth; the second, 0.8 c.c. of broth and 0.2 c.c. of normal serum; the third, 0.9 c.c. of broth and 0.1 c.c. of normal serum; and the fourth and fifth, the corresponding amounts of immune rabbit serum and broth. The tubes of each series were inoculated with the same quantity of bacterial culture, incubated, and plates were made at the end

of four to six hours, and again after 20 to 24 hours.* In some instances a normal rabbit serum would prove as strongly bactericidal as the immune serum if a large amount was used, say one part of serum to five of broth, and plates made only at the end of four hours; but if the two sera were diluted to 1:10 or 1:20, a decided difference always appeared in favor of the immune serum, and the 24 hour plates from all tubes usually showed that the action of the normal serum was transient.

Table 1 shows typical results in the use of the immune and normal sera.

The immune serum contains an agglutinin for these bacilli, yet the agglutination test is of little value in identifying the members of this group, owing to the fact that so many of them tend to clump in fluid suspension. Even a homogeneous suspension will often change after several hours in the incubator, and the tubes which contain no serum will appear as strongly agglutinated as those with serum. Especially is this true of strains which resemble closely *B. diphtheriae*, the so-called "diphtheria-like" bacilli. There were, however, several strains (Nos. 4, 5, and 9) which responded well to the agglutination test. Our immune rabbit serum agglutinated these in dilutions of 1:1,000 up to 1:5,000 according to the strength of the serum.

In the experiments given above, rabbit serum was used exclusively, but as it was desirable to obtain larger quantities of immune serum than rabbits could furnish, we immunized a goat also. The serum of goats is normally bactericidal, to these organisms more uniformly and in higher degree than is the serum of rabbits,† and the bactericidal substance increases very slowly with immunization. On the other hand, the agglutinins appear early and increase rapidly, so that the serum of an immune goat, which is no more highly bactericidal than it was before immunization began, is already strongly agglutinative, while the serum of an immune rabbit, which has increased more than tenfold in bactericidal power, is only moderately agglutinative.‡ We have had goat serum which agglutinated No. 9 in dilutions of 1:30,000, but no rabbit serum has ever agglu-

*As some of the strains agglutinate with the immune serum, it was thought that inaccurate results might come from using simply a loopful for plating, and we therefore tried plating the whole contents of the tube; but our results in these experiments did not differ from those obtained by the former method.

†Our work with normal goat serum is not yet complete, and we can only say that typical *B. diphtheriae* is not killed by normal goat serum. There are, however, pseudodiphtheria bacilli not belonging to our group which are killed by goat serum.

‡It is evident that in the case of immune rabbit and goat serum it is not true that agglutinative power and protective power increase side by side and in relative degree as has often been asserted of other bactericidal sera.

TABLE 1.

EFFECT OF SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED WITH VIRULENT PSEUDODIPHTHERIA BACILLUS UPON VIRULENT PSEUDODIPHTHERIA, DIPHTHERIA, AND ORDINARY OR NONVIRULENT PSEUDODIPHTHERIA BACILLI.

BACILLI	NORMAL RABBIT SERUM				IMMUNE RABBIT SERUM						CONTROL	
	Serum 0.2 c.c. Broth 0.8 c.c.		Serum 0.1 c.c. Broth 0.9 c.c.		Serum 0.2 c.c. Broth 0.8 c.c.		Serum 0.1 c.c. Broth 0.9 c.c.		Serum 0.05 c.c. Broth 0.95 c.c.		Broth	i.c.c.
	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.		
Virulent pseudodiphtheria bacillus (Ruediger No. 3, from throat of scarlet fever).....	6,000	∞	6,000	∞	0	1	27	35	50	496	∞	∞
Virulent pseudodiphtheria bacillus (Ruediger No. 4, from throat of scarlet fever).....	4,224	∞	5,000	∞	24	1	34	6	26	0	4,000	∞
Virulent pseudodiphtheria bacillus (Hamilton No. 11, from throat in laryngitis).....	400	7,000	200	7,100	25	700	8	600	30	330	560	6,000
Virulent pseudodiphtheria bacillus (Hamilton No. 16, from scarlatinal urine).....	2,000	∞	2,510	∞	0	0	200	0	8,000	∞
Typical diphtheria bacillus (from diphtheric throat).....	23	∞	21	∞	10	∞	37	∞	17	∞
" " (" ").....	570	3,223	110	1,616	800	3,440	750	3,880	850	4,160	100	440
"Diphtheria-like" bacillus (A. Williams No. 9).....	1,760	∞	1,840	∞	1,600	∞
Ordinary pseudodiphtheria bacilli No. 20 otitis media....	560	600	840	720	440	2,000	1,000	4,500	10,000
" " " No. 27 conjunctivitis....	600	∞	400	∞	350	∞	350	∞	480	∞	8,000	∞
" " " No. 31 scarlatinal throat....	800	∞	8,000	∞	8,000	∞	8,000	∞	8,000	∞	750	∞
" " " No. 22 " ".....	1,440	∞	880	∞	1,280	∞	720	∞	2,000	∞

tinated in dilutions higher than 1:5,000. The immune goat serum, however, has so far not attained the bactericidal power of some rabbit sera. We have therefore used rabbit serum usually in testing the bactericidal action on cultures of diphtheria-like and pseudodiphtheria bacilli, but in the study of the nature of the bactericidal substance we have used both goat and rabbit serum, and, in absence of a statement to the contrary, our conclusions apply to both kinds of serum.

All our experiments with immune serum, both of rabbits and of goats, have shown that the bactericidal substance is stable, resisting the deteriorating effects of age, light, heat, and even drying, to an unusual degree.

Deterioration from age takes place much more slowly when the blood is allowed to clot and the serum left in contact with the clot, than when it is removed by defibrinating and centrifuging the blood. To obtain a serum strongly bactericidal it is necessary to let it remain in contact with the clot for at least four hours. If it is to be kept for some time, it is better to remove it from the clot only as needed. By this method the serum, kept in contact with the clot in the ice-box, was found to be bactericidal after 32 days. This fact, that the presence of the clot postpones the deterioration of a bactericidal serum, has been noted by Ainley Walker, who also succeeded in reactivating old serum with the serum-free extract of fresh blood-clot. He concludes that "complement is formed by leucocytes and becomes liberated into plasma or serum by their disintegration," and thus the disintegrating leucocytes supply continually fresh complement and prevent deterioration of the serum. Whether or not Walker's explanation of the phenomenon is correct, we have found his statement to be true in the case of our immune serum.

One week's exposure to strong daylight, not necessarily sunlight, weakens, but does not entirely destroy, the bactericidal power of these sera.

Zero temperature partially inhibits the action of the bactericidal sera, but not entirely. The usual mixture of serum and culture was placed in a tube, and the tube thrust into a pan of powdered ice. A loopful plated at once gave 8,000 colonies, while the same dilution at 37° C. gave only 388. The same serum, however, became active again

when placed in the incubator, and a plate from the same tube, at the end of 24 hours at 37° C., had only 720 colonies. If the bacilli are removed from the serum, washed, and then incubated, they will be found to have absorbed the bacteriolysin while at 0° C.—a point which will be referred to later on.

Filtration through a porcelain filter removes the active principle concerned in bacteriolysis and the filtrate is inactive, provided a very fine filter is used, otherwise a part of the bactericidal substance will pass through. The bacteriolysin of normal goat serum can be removed in the same way.

The serum can be evaporated to dryness without losing its bacteriolytic power.

When we proceeded to test the effect of temperature, we found that the resistance to heat, like the resistance to age, is much greater than that usually found in bactericidal serum. Room temperature is well borne, provided the serum is protected from light, and a week's exposure to 37° C. is not sufficient to produce complete inactivation. Exposure to 58° C. for 30 minutes has no effect upon the bactericidal power, and to 65° C. for 30 minutes produces only a slight loss. When, however, the time of exposure is increased to two hours, a gradual loss of power occurs beginning at 60° C. and continuing to the point at which the serum, diluted 1 : 4 with normal salt solution, coagulates, about 85° C.

Somewhere between 80° and 90° C. the bactericidal substance is destroyed. Two hours' exposure to 80° C. is not always sufficient to destroy it in perfectly fresh, strongly bactericidal serum, but an hour's exposure to 85° C. coagulates the diluted serum, and the fluid expressed from the coagulum is inactive. Serum heated to 70° C. for two hours is no longer capable of protecting guinea-pigs against a lethal dose of pseudodiphtheria bacilli.

All these experiments, except the last mentioned, were made with both goat and rabbit serum, and the two gave the same results. The normal serum of both these animals differs from the immune in resistance to heat, losing its bactericidal power by exposure to 70° C. for two hours. The difference in heat resistance of normal and immune serum has been noted before. Ehrlich and Morgenroth¹

¹ *Gesammelte Arbeiten zur Immunitätsforschung*, Berlin, 1904, p. 21.

found, for instance, that the hemolysin for sheep's corpuscles present in normal goat serum was destroyed at 57° C., but the hemolysin in the serum of a goat immunized with sheep's blood could resist 65° C. with only a slight diminution of its power. This heat-resisting complement was by them supposed to be present in normal goat serum, in small amounts, and to be increased by immunization.

The possibility must always be considered that a bactericidal serum, which is very resistant to high temperature, owes its power to a strong alkaline reaction. Thus Hamburger has increased the alkalinity of blood by passing CO₂ through it, and found it to be more strongly bactericidal in consequence. Emmerich claims that he has reactivated inactivated dog serum with sodium hydroxide, and Fodor¹ believes that the increased alkalinity of rabbit serum after immunization against anthrax is responsible for its bactericidal power. However, in the case of our immune serum, increased alkalinity can be ruled out as a factor in the increased bactericidal power, for titration shows that there is no difference between the reaction of normal and that of immune rabbit serum.

To sum up then : The immune serum is not more alkaline than is normal serum. It is very resistant to heat, to room or incubator temperature, to age, to cold, and to drying. It is partly or wholly inactivated by filtration. The question as to the exact nature of the bacteriolytic substance is not easy to answer, but we incline strongly to the view that the active principle is a single substance rather than complement and amboceptor. We have never been able to separate the active principle into two bodies by any method thus far attempted. The high degree of heat resistance and of resistance to age certainly argues against the presence of complement, especially when it is found impossible to reactivate the heated serum or the old serum. Our experiments in reactivation have been unsatisfactory, owing somewhat, perhaps, to the fact that both heated serum and normal unheated serum are bactericidal to a slight degree, and therefore it is difficult to estimate results. We have tried repeatedly to restore the complement to heated serum, but have never yet obtained indubitable results. It might be argued that the intermediary body also is destroyed at 80° C., and that this is the explanation of the failure of

¹ References given in KOLLE U. WASSERMANN, *Hb. d. path. Mikro-Organ.*, 1904, Bd. IV, p. 560.

reactivation experiments, but that would be to assume equal stability in complement and amboceptor ; indeed, it would necessitate an entirely new conception of complement.

The attempt to remove the amboceptor by absorption resulted in the absorption of the bactericidal substance *in toto*, just as the effort to inactivate by heat resulted in permanent inactivation. Bacilli were treated with immune serum at zero, were then centrifuged, washed three times, and placed in the incubator, some with normal salt solution only, others with different quantities of normal rabbit serum. There was no difference in the plates made from the tubes, except what could be accounted for by the slight bactericidal action of the normal serum.

In a recent study of the anthracidal substance in the serum of the white rat made by one of us,¹ the properties of the anthracidal substance in the serum of adult rats were found to correspond in several ways with those of our bactericidal serum. It was found that, while the anthracidal substance in the serum of young rats is thermolabile, the same cannot be said of the substance in the serum of older rats. In the latter the anthracidal substance is very stable, resisting exposure to a temperature of 66° C., resisting direct daylight for nine days, and the temperature of the incubator for three weeks, before complete destruction, and the temperature of the ice-box for the same period without any loss of power at all. The activity is partially suspended at 0° C. Alkalinity seems to have nothing to do with bacteriolysis, for the heated and unheated sera have the same degree of alkalinity. The conclusion drawn as a result of these observations was the same as that we have sustained in regard to our goat serum and immune rabbit serum ; namely, that the anthracidal substance in the serum of adult rats is simple and indivisible.

Our experiments with the immune serum in regard to its effect upon phagocytosis confirm the conclusions reached in the experiments in bacteriolysis outlined above, as they show that we have here a specific serum which acts in a certain way toward the organisms belonging to the same group as the one used to produce immunity, and not toward other pseudodiphtheria bacilli or toward the diphtheria bacillus. The experiments also indicate that phagocytosis

¹ HORTON, *Jour. Infect. Dis.*, 1906, 3, p. 110.

plays an important rôle in the protection of guinea-pigs against these pseudodiphtheria bacilli.

In the course of our experiments on the action of immune serum in guinea-pigs injected with a lethal dose of *B. No. 4*, we had occasion to examine the blood of these animals and compare it with that of normal guinea-pigs, and with that of guinea-pigs injected with the same amount of culture, but without immune serum. When two guinea-pigs are given each a fatal dose of *B. No. 4*, and guinea-pig B is given a dose of protective serum, while A is not, the blood of A will show an increase of polymorphonuclear leucocytes as compared with the blood of a control animal, but the blood of B will show a far greater increase as made clear in the following differential count.

DIFFERENTIAL BLOOD COUNT.

	Per Cent Polynuclear	Per Cent Small Mono- nuclear	Per Cent Large Mono- nuclear
Control Guinea-pig.....	1.6	82.0	16.0
Guinea-pig A-2.5 c.c. broth culture <i>B. No. 4</i>	8.0	87.0	4.0
Guinea-pig B-2.5 c.c. broth culture <i>B. No. 4</i> + 2 c.c. immune serum.....	44.0	44.0	11.0

Such an increase in the number of polymorphonuclear leucocytes as a result of the injection of the immune serum points certainly to an increased leucocytic activity under the stimulus of the serum.

That phagocytosis is dependent upon a substance in the serum has been shown by Wright and Douglas. In the study of the effect of our immune serum on phagocytosis the technique followed was the same as that followed by Hektoen and Ruediger.¹

The blood was drawn by means of a sterile glass syringe directly from the heart of the lower animals, and from the vein at the bend of the elbow in human beings. The washed corpuscles were added to tubes containing serum and a suspension of bacilli in normal salt solution, in the proportion of 0.25 c.c. of corpuscles to 0.25 c.c. of serum and 0.5 c.c. of bacterial suspension. We used the corpuscles of the dog, goat, guinea-pig, and rabbit — in the last case obtaining polymorphonuclear leucocytes by injecting aleuronat suspension into the pleural cavity and withdrawing the exudate—and also of 12 healthy adults. In each instance control tubes were prepared, one containing no serum at all, only normal salt solution; the second containing normal rabbit serum, and to these was often added a third tube containing the serum homologous to the corpuscles. After incubation for one hour at 37° C., smears were made from the tubes, stained with Leishmann's stain, and examined for phagocytes.

¹ *Jour. Infect. Dis.*, 1905, 2, p. 128.

Nine experiments were made with leucocytes of lower animals, and 40 with human leucocytes. Without exception, in all of these experiments the presence of our immune serum favored phagocytosis of this group of bacilli, but had no effect on phagocytosis of *B. diphtheriae*, or of six strains of ordinary pseudodiphtheria bacilli. There was practically no phagocytosis in the tubes containing no serum, there was a varying amount in the tubes with homologous serum, but in the experiments with the virulent pseudodiphtheria bacilli there was always a marked increase in the amount of phagocytosis in the tubes with immune serum as compared with normal rabbit serum. Table 2 shows this, the results being stated in figures which indicate the average number of bacilli found in a count of 40 polymorphonuclear leucocytes.

TABLE 2.

PHAGOCYTOSIS OF VIRULENT PSEUDODIPHTHERIA BACILLUS UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Corpuscles in NaCl Sol.....0.25 c.c. Serum or NaCl Sol.....0.25 c.c. Bacterial Suspension.....0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)			
	NaCl Sol.	Homologous Serum	Normal Rabbit Serum	Immune Rabbit Serum
Guinea-pig leucocytes (blood).....	0.1	1.5	1.5	5.5
Goat " ".....	0.1	1.9	1.5	9.5
Rabbit " (exudate).....	1.8	..	6.0	7.8
Dog " (blood).....	1.0	3.5	13.5	22.0
Human " ".....	2.8	11.4	4.3	20.7

Immune goat serum also causes marked phagocytosis of virulent pseudodiphtheria bacilli by human leucocytes, while normal goat serum had but little opsonic effect, the average in three different experiments not exceeding 1.7 per leucocyte.

In most of the experiments *B. No. 1*, which had been used for immunization, was used also for testing phagocytic power, but it was observed that the same results were obtained with *B. No. 4* (Ruediger) and *Nos. 5, 9, 11, and 16* (Hamilton). It must be added that the sera of five immune rabbits and of more than a dozen non-immune rabbits were used in these experiments, so that an individual peculiarity in the serum of any one animal can be ruled out.

An identical series of tubes was prepared at the same time, and a suspension of *B. diphtheriae* was substituted for the pseudodiph-

theria bacillus, in order to ascertain whether the opsonin present was specific for the latter. The result is illustrated in Table 3:

TABLE 3.

PHAGOCYTOSIS OF DIPHTHERIA BACILLI UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Corpuscles in NaCl Sol. 0.25 c.c. Serum or NaCl Sol. 0.25 c.c. Bacterial Suspension. 0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)			
	NaCl Sol.	Homologous Serum	Normal Rabbit Serum	Immune Rabbit Serum
Guinea-pig leucocytes (blood)	0.0	1.8	0.6	0.87
Goat " "	0.0	4.4	2.3	3.0
Dog* " "	9.9	40.0	40.0	40.0
Human " "	1.4	7.8	4.0	2.7
Human* " "	0.5	40.0	40.0	40.0

* Leucocytes so filled with bacilli that no accurate count could be made.

As shown in Table 3, there is no difference in the opsonin of immune serum and that of normal serum as regards diphtheria bacilli.* Similar results were obtained with six strains of typical pseudodiphtheria bacilli (Table 4). The opsonin is therefore specific for the virulent group of pseudodiphtheria bacilli, just as the bactericidal substance is specific for them. It is well in this connection to emphasize the fact that the same effect is produced in both animals, the goat and the rabbit, by immunization. In the serum of both there appears a specific opsonin and a specific bacteriolysin which have the same degree of thermostability and of resistance to age and light.

TABLE 4.

PHAGOCYTOSIS OF TYPICAL, NON-VIRULENT, PSEUDODIPHTHERIA BACILLI UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Human Corpuscles in NaCl Sol. 0.25 c.c. Serum or NaCl Sol. 0.25 c.c. Bacterial Suspension. 0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)		
	NaCl Sol.	Normal Serum	Immune Serum
No. 20, diphtheria—ear.	0.1	5.00	4.4
No. 27, conjunctivitis—eye.	0.2	1.5	0.85
No. 34, measles—ear.	0.7	4.2	4.7
No. 31, scarlet fever—throat.	0.0	0.45	0.9
No. 6, measles—throat.	0.7	11.0	4.0

*These results obtained with *B. diphtheriae* are not in accordance with those of Wright and Douglas who state that *B. diphtheriae* is not taken up by human leucocytes, but they are in accordance with the results recently published by Walkerr (*Jour. Med. Res.*, 1905, 13, p. 173). In his experiments—made chiefly with the leucocytes of one individual—there was always phagocytosis of *B. diphtheriae*, although never to the extent shown in two of our cases. The individual variation is apparently very great in the case of *B. diphtheriae*.

In course of these experiments several points of difference have appeared between the bactericidal substance in our sera and the opsonin, showing that they are not identical. In the first place opsonin disappears almost completely after heating immune rabbit serum at 70° for two hours and from immune goat serum after heating at 70° degrees for one hour, while the bacteriolysin persists in immune serum heated at 80°. This persistence of the bacteriolysin makes it impossible to prove destruction of bacilli by phagocytosis *in vitro* as might be done were the bacteriolysin destroyed at a lower temperature than the opsonin instead of a higher. In the second place, a serum strongly bactericidal may have little opsonin. Normal goat serum is rich in bacteriolysin, but has almost no opsonin, while immune goat serum has acquired a large amount of opsonin without any increase in bacteriolysin. Normal human serum, which is only slightly bactericidal, may contain a large amount of opsonin.* So far as our experiments go, it appears that the opsonin for these bacilli is not composed of a complement-amboceptor complex.

Just as the bacteriolysin in normal goat serum was found to be less resistant to heat than that in immune serum, so the opsonin in normal serum is less resistant than that in immune serum. For a perfectly satisfactory proof of this one would have to compare two specimens of rabbit or goat serum, normal and immune, but these normal sera contain so very little opsonin that no striking results can be obtained with them. The opsonin in human serum is destroyed at a far lower temperature than that in the serum of immune goats and rabbits; namely, at 56° to 58° C. for 30 minutes.†

TABLE 5.
THE EFFECT OF HEAT UPON THE OPSONIN FOR VIRULENT PSEUDODIPHTHERIA BACILLI.

Mixtures		Phagocytosis (40 Leucocytes Counted)
Bacterial suspension 0.5 c.c. + washed human corpuscles 0.25 c.c. +	Immune rabbit serum 0.25 c.c.	16.3
	Heated at 70° C. 2 hrs. 0.25 c.c.	1.8
	Immune goat serum 0.25 c.c.	11.7
	Heated at 70° C. 1 hr. 0.25 c.c.	1.0
	Normal human serum 0.25 c.c.	19.3
	Heated at 56° C. 30 min. 0.25 c.c.	1.65

* We have not tested many specimens of human serum for bacteriolysin, and are unable to say whether or not there is a decided individual variation in this respect.

† We have not tested the heat resistance of the opsonin for virulent pseudodiphtheria bacilli by Dean's method, *Proc. Roy. Soc.*, 1905, Series B, 76, p. 506.

NATURE OF THE PROTECTIVE ACTION OF IMMUNE SERUM.

In view of the unvarying results obtained in the test-tube experiments in phagocytosis, it would seem probable that the same process occurs in the animal body and that the immune serum protects guinea-pigs against the virulent pseudodiphtheria bacilli by increasing phagocytosis as well as by direct bacteriolysis. We have said that guinea-pigs, dying after injection with a fatal dose of virulent pseudodiphtheria bacilli, yield growths of the organism from peritoneal and pleural fluids, from urine, heart's blood, lungs, liver, spleen, and kidneys. Three hours after inoculation this general invasion has taken place, for cultures made at this time all yield growths. This is true of animals protected by immune serum as well as of those not so protected. If both animals are killed at the end of three hours, a general infection will be found to have occurred in both, but the number of bacilli in smears from the peritoneal exudate of the one which received protective serum is not nearly so great as in the case of the unprotected guinea-pig, and the bacilli are agglutinated. After three hours a progressive diminution of bacteria occurs in the body of the protected guinea-pig. In the animals killed at the end of six hours, cultures are obtained from the spleen and peritoneal cavity only; and in those killed at the end of 18 hours all cultures remain sterile. The bacilli evidently multiply rapidly and invade the general circulation within three hours after inoculation, even in the presence of protective serum. Destruction of bacilli has begun, however, in the presence of immune serum, during the first three hours, and from then on proceeds rapidly, being well advanced at the end of six, and complete at the end of 18, hours. The results of experiments in the test tube appear to correspond with those in in the animal body.

TABLE 6.

 PROGRESSIVE BACTERIOLYSIS *in vitro*.

Suspension of <i>B. No. 6</i>	0.75
Immune serum	0.25
One loopful plated at once	33
" " " after 1½ hrs.	1,200
" " " " 3 "	800
" " " " 4½ "	560
" " " " 6 "	52
" " " " 18 "	36

We have already spoken of the increase of polymorphonuclear leucocytes in the blood of protected guinea-pigs as compared with those not so protected. There is also a much greater number of leucocytes in the peritoneal fluid of protected animals, as shown in the following experiment:

Guinea-pig A was killed three hours after receiving intraperitoneally 4 c.c. of a broth culture of *B. No. 4*. The exudate was almost clear with many bacilli and but few leucocytes, 2 per cent of which were polynuclears, 94 per cent small mononuclears, and 4 per cent large mononuclears.

Guinea-pig B received, in addition to 4 c.c. of broth culture of *B. No. 4*, 2 c.c. of immune serum. It was killed at the end of three hours. The peritoneal exudate was slightly cloudy, and contained few bacilli and many leucocytes, 54 per cent of which were polynuclears, 28 per cent small, and 12 per cent large mononuclears.

So large an increase of polymorphonuclears in the blood and in the exudate certainly would seem to point to phagocytosis, but we must acknowledge that we were never able to demonstrate any considerable number of phagocytes in smears from the blood or from the organs of our protected guinea-pigs; in the peritoneal exudate, however, quite marked phagocytosis takes place.

An animal which was given immune serum and a lethal dose of *B. No. 4*, was killed at the end of six hours. Cultures showed that bacilli were present only in the spleen and peritoneal fluid. Smears from the peritoneal fluid showed no active phagocytes at all; smears from the spleen showed that of 75 polymorphonuclear leucocytes only 13 were engaged in phagocytosis, and these contained an average of four bacilli to the phagocyte.

Another guinea-pig was given the usual dose of broth culture and immune serum, and a small amount of fluid was withdrawn from the peritoneal cavity by means of a syringe every 30 to 40 minutes for two and a half hours after injection.

The following results were obtained:

After $\frac{1}{2}$ hour	no polymorphonuclear leucocytes
" 1 "	average of 20 leucocytes = 8.7 bacilli
" 1 hr. 40 min.	" " " = 6.7 "
" 2 hrs. 25 "	" " " = 13.4 "

A second guinea-pig treated in the same way at the same time was killed at the end of three hours. The peritoneal fluid contained 54 per cent of polymorphonuclear leucocytes, but they averaged only 1.9 bacilli to the polymorphonuclear leucocyte. There were no phagocytes in the blood or in the smears from any organ except the spleen, where a few were seen.

Phagocytosis in the peritoneal cavity is apparently at its height about two hours or two hours and a half after injection, and the average of bacilli found so far in the leucocytes at that time is 13 to the polymorphonuclear leucocyte. Now the almost complete

absence of phagocytosis in the organs and in the blood, which contained bacilli as shown by cultures, might appear to throw some doubt upon the importance of the part played by phagocytes in the action of our immune serum, at least so far as ridding the blood of bacilli is concerned. It may be, however, that we have not yet found the right moment for making search for phagocytes. It should be noted that so far we have neglected to study carefully the bone marrow from this point of view.

SUMMARY.

1. The bacilli described by us under the name of "virulent pseudodiphtheria bacilli" form a group distinct from *B. diphtheriae* on the one hand and from other pseudodiphtheria bacilli on the other.

2. The distinction lies in the fact that goats and rabbits, immunized against one member of this group, yield serum which is bactericidal to the others of the group, but not to *B. diphtheriae* or to other pseudodiphtheria bacilli, and which contains opsonin specific for the members of this group.

3. The study of the bacteriolysin and opsonin of our immune serum reveals facts which should be of general interest to immunologists, inasmuch as we have here an immune serum of high degree of thermostability, apparently not containing complement and amboceptor.

4. Experiments on guinea-pigs indicate that the immune serum causes a marked polynucleosis, and increases phagocytosis *in vivo* as well as *in vitro*; hence the protective action of this serum must be ascribed in part at least to the immune opsonin.

6. The bacteriolysin and opsonin of these immune sera are not the same substance.

In the future we hope to revert to the question as to the frequency of "virulent pseudodiphtheria bacilli," and to take up again their clinical significance.

THE IDENTITY OF FUSIFORM BACILLI AND SPIRILLA.*

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It has been observed for several years that fusiform bacilli, usually associated with long spirilla,† are present in many pathological conditions. They were first demonstrated in cases of ulceromembranous angina (Vincent's or Plaut-Vincent's) and later in cases of ulceromembranous stomatitis, hospital gangrene, noma, pyorrhea alveolaris, appendicitis, subpectoral, brain, and thigh abscesses, and some other morbid processes. Both organisms have been observed in the healthy mouth and genitalia. In the majority of cases the bacilli and spirilla have been recognized in smear preparations, made from the seat of the disease, or in stained sections of tissues. Both organisms have been grown in mixed cultures. Fusiform bacilli have been isolated in pure culture by Veillon and Zuber, Ellermann and Weaver. The fusiform bacilli isolated by Dr. Weaver, and described in an article by Dr. Weaver and myself,¹ appeared to be the same as those described by Ellermann, and compared quite closely with those found in smear preparations made from the seat of the disease. Although spirilla resembling those from the tissues have been grown in mixed cultures with fusiform bacilli and other organisms, all efforts to obtain them in pure culture have up to this time been unsuccessful.

Most authors have believed that the fusiform bacilli and spirilla are entirely distinct organisms and that they act in symbiosis, the spirilla serving to increase the virulence of the bacillus. Some observers have maintained that they are different forms of one organism (Seiffert, Perthes, Sobel, and Herrman). As until recently no pure cultures of the bacilli and none of the spirilla have been obtained, it has been impossible to prove whether they were distinct organisms or not.

*Received for publication January 2, 1906.

† The term "spirilla" and "spirochetæ" have been used indiscriminately in the literature on this subject.

¹ *Jour. Infect. Dis.*, 1905, 2, p. 446.

PLATE 2

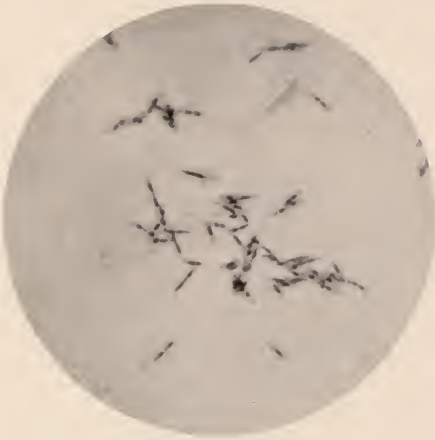


FIG. 1.

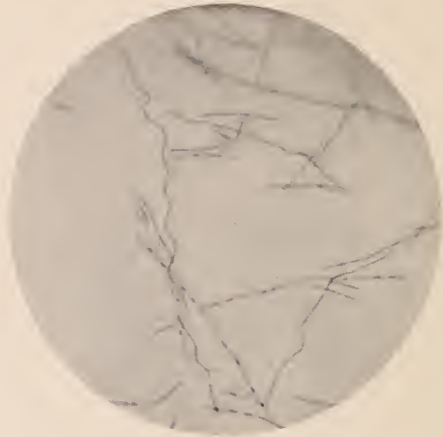


FIG. 2.



FIG. 3.



FIG. 4.

While studying some pure cultures of fusiform bacilli, it was observed that after they had grown from 48 hours to five days, spirilla were formed which greatly resembled those seen in smear preparations made from the gums and from the various pathological lesions in which spirilla had been observed. No such spirilla had been seen in the cultures previously described by Dr. Weaver and myself, but on examination of some of these old, dead cultures, spirilla were found similar to those observed in the living cultures. The spirilla could be easily overlooked, as often they are present for only a few days in any considerable number, and are more readily seen when stained by carbol-gentian-violet than by carbol-fuchsin, which was usually employed in the earlier cultures. Ellermann states that no "spirochetæ" were present in his pure cultures of fusiform bacilli.

The three organisms which are here described were isolated from the gums of healthy mouths. In one case there was considerable tartar around the teeth, which were badly decayed. In smear preparations made from the gums the fusiform bacilli and spirilla corresponded to those found in the pathological lesions in which these organisms are associated. Their morphology does not need any description at this time. The material from the gums was smeared over the surface of a series of slants of ascites agar (1:3). After anaerobic incubation at 37° for two or three days the growth of fusiform bacilli appeared in delicate, whitish colonies, 0.5-2 mm. in diameter, resembling colonies of streptococci. By inoculation from these colonies pure cultures were obtained. In one case the first two tubes contained streptococci and fusiform bacilli, and the third, fusiform bacilli alone.

CULTURAL PROPERTIES.

The organisms are obligate anaerobes growing at 37°, but not at room temperature. The organisms show no progressive motion. They possess considerable vibratory motion, mostly at the extremities, but sometimes it extends from one end to the other. The organisms were examined by Dr. D. J. Davis with the ultramicroscope, and this motion was seen to be similar to that of dead cilia in cerebrospinal fluid, as observed by him.¹ As in the case of the cilia, the motion probably depends upon physical factors. With the ultramicroscope spirilla from the gums were seen to possess no progressive, but a decided cork-screw and lateral motion. This lateral motion is probably similar to the vibratory motion described above. No flagella have

¹ *Trans. Chicago Path. Soc.*, 1904, 6, p. 225.

been demonstrated. Johnston's staining method, which was employed, readily stained typhoid flagella.¹

The cultures were grown by Wright's method, by saturating the cotton stopper with a strong solution of pyrogallic acid in a 5 per cent solution of sodium hydroxide, and closing the tube with a tightly fitting cork, sealed with paraffin.

Slants of ascites-agar.—At the end of 24 to 48 hours a delicate, whitish, irregular growth appears, usually with delicate colonies along the edge from $\frac{1}{2}$ to 2 mm. in diameter. A flocculent deposit collects at the bottom of the fluid of condensation.

Loeffler's blood-serum.—In 24 to 48 hours a slightly moist, irregular growth appears, with colonies along the border. A flocculent deposit collects at the bottom of the fluid of condensation.

Rabbit's blood-agar (1:4).—The growth is similar to the preceding but of a brownish color.

Agar slant.—In 24 to 48 hours a very delicate, whitish growth forms with small colonies at the border.

Glycerin-agar slant.—There is no growth.

Glucose-agar stab.—A delicate, whitish growth with small lateral prolongations develops along the needle track in 24 to 48 hours. A little gas is usually formed.

Stab in glucose-agar and ascites-fluid (3:1).—The growth is similar to the preceding, but somewhat more abundant. A little gas is formed.

Litmus milk.—In 48 hours there is a moderate growth, the medium being decolorized. There is no coagulation. After oxygen is admitted the medium assumes its original color.

Potato.—There is no growth.

Dextrin-free broth.—No growth occurs.

Plain nutrient broth.—There is no growth. Growth does not occur in broth containing 2 per cent glucose, but does occur if it contains between 0.25 and 1 per cent. The growth appears in 24 to 48 hours. It settles somewhat to the bottom, but the fluid is turbid throughout.

Ascites broth (1:3).—A slight, flocculent growth appears in 24 to 48 hours. It settles in a thick mass to the bottom, leaving a clear fluid above. On shaking, the growth may be separated into visible granules.

NaCl agar (2 per cent).—No growth.

NaCl broth (2 per cent).—No growth.

NaCl ascites broth (2 per cent).—No growth.

In all of the successful cultures a somewhat offensive odor is given off.

The fusiform bacilli described by Dr. Weaver and myself grew on glycerin agar, and in dextrin-free broth. They did not grow in milk. Although there are these differences between those cultures and the ones here described, the variations are too slight to conclude that the organisms are not the same. The number and age of the bacilli inoculated are important and may account for the variations in results. However, it doubtless will be found that there are several varieties of fusiform bacilli, since those already isolated differ somewhat from one another.

¹ *Loc. cit.*, 1905, 2, p. 343.

The organisms have retained their vitality 55 days, the length of time which has elapsed since their isolation. It seems to be true that later generations of these organisms have considerably less vitality than the organisms first isolated. The factors which especially influence the formation of spirilla have not yet been ascertained. Further cultivation of the organisms will probably show the best conditions for their development. Occasionally a culture is found in which short bacilli only are present. Sometimes no growth occurs after the first 24 or 48 hours, which may account for the absence of filaments and spirilla. Filaments and spirilla are formed as late as the 10th generation, the generation now under observation.

MORPHOLOGY AND STAINING PROPERTIES.

The organisms present the same morphological appearance in whatever media grown. They are extremely polymorphous, appearing as quite different organisms at different periods of their development. They are, usually during the first 24 hours of their growth, delicate, pointed rods from 3 to 10μ in length. As a rule they show deeply staining bodies or bands, most often two in number, and not situated at the ends. The bacilli are usually straight, but sometimes bent. The bacilli often strikingly resemble barred forms of diphtheria bacilli. They are often slightly larger in the center, but not always. In these young cultures a few smaller, 1.5 to 4μ in length, and plumper bacilli are also sometimes found. They have very thick unstained bodies, with deeply stained rounded ends. The swollen bodies resemble spores, but do not stain as such. Both forms appear in pairs, end to end, at obtuse angles, and in rows.

In some of the longer bacilli, usually during the first days of their growth, a few spores are seen. There is usually one in a bacillus, but occasionally there are two. They are situated either at one extremity or near the center. They may be seen within, or partly without, or entirely outside, the bacillus. The development from the round spores into the very short, plump bacilli with dark extremities may be observed in a hanging drop. The various stages can also be found in stained preparations. The spores are best seen when stained with carbolfuchsin. They retain the stain when treated as tubercle bacilli and decolorized with 1 per cent sulphuric acid solution in water.

In 24 to 48 hours, or even later, filaments of various lengths are formed. Some of the filaments are of the same diameter throughout and contain, as a rule, deeply staining bodies sometimes round, oftener like bands. Similar ribbon-like forms are frequently seen in smear preparations from the gums. Some of the filaments stain uniformly. As a rule many of the filaments are seen to be made up of strings of bacilli, which are joined at the dark bodies. The bacilli forming the filaments vary in size and shape as the bacilli in the earlier cultures do.

The filaments are sometimes straight, sometimes wavy. Involution forms in a great variety of shapes are frequently observed. In the older cultures the filaments often stain irregularly. Clear spaces resembling vacuoles are occasionally seen. They are simply the unstained bodies of the short, plump bacilli, which in chains are so close together as to appear like vacuoles.

Soon after, or simultaneous with, the appearance of the filaments, most often on the fourth or fifth day, spirals are observed, sometimes in enormous numbers. As a rule they stain uniformly; others show the dark bodies seen in the short bacilli and filaments. Often it is easily seen that the spirilla are made up of chains of short bacilli similar to the straight filaments. The spirals are sometimes in the form of cork-screws, more often the turns are not so sharp, nor so deep. They form from one to twenty curves. The turns are sometimes rounded, sometimes very pointed. The pointed ones are especially marked when the spirilla can be seen to be made up of bacilli and when there is only one bend. Some of the longer spirals extend across the whole field, more often they are shorter, showing four or five turns. These shorter forms are from 5 to 10 μ in length. They vary considerably in the depth of the curves as do the uncultivated spirilla. The ends are usually pointed. Toward the extremities the curves sometimes become more and more broad. Involution forms are seen in the spirilla as well as in the filaments. In some of the cultures the spirilla alone are found, but usually filaments and short bacilli are also present. By the 10th to the 15th day fewer filaments and spirilla are seen, but as a rule, even in the older cultures (55 days), spirilla can still be found.

Both the bacilli and spirilla stain by methylene blue, gentian-

violet, Giemsa, Romanowsky, carbolfuchsin and carbol-gentian-violet. The last stain was found to be the most satisfactory for staining the spirilla. A solution was used containing 1 c.c. of alcoholic gentian-violet and 10 c.c. of a 5 per cent carbolic acid solution. The smear preparation is allowed to dry in the air. Without pre-



FIG. 5.—Drawing shows spirilla of different shapes seen in four-day-old culture on ascites agar. X about 1000.

viously fixing the specimen, the stain is dropped on and is allowed to boil. The preparation is washed in water, dried, and mounted. The deeply staining bodies appear with all the stains. Neither the bacilli nor the spirilla stain by Gram's method. That the spirilla are not artefacts follows from their appearance in hanging-drop preparations, their appearance in stained preparations, whether fixed by heat or not, and when

stained with all the various stains previously mentioned, and from their occurrence with the various culture media in which the bacilli grew.

INOCULATION EXPERIMENTS.

Cultures containing short forms and also cultures containing spirilla were injected subcutaneously and into the muscles of guinea-pigs without any result up to this time.

The cultivated fusiform bacilli and spirilla were compared with the fusiform bacilli and spirilla as found in smear preparations from 35 different sources: three from cases of ulceromembranous angina; two from cases of combined ulceromembranous angina and stomatitis; one from a case of noma; 14 from tartar and gums of healthy mouths. The artificially cultivated and uncultivated organisms closely resemble each other in their morphological and staining properties. All the variations, which are many, in form and size, seen in the uncultivated organisms are to be found in these cultures.

In certain fields the spirilla and fusiform bacilli present pictures strikingly similar to those seen in smears made direct from the mucous surfaces.

The bacilli have been described as non-motile, both in fresh specimens and in cultures. The uncultivated spirilla seem to vary in the degree of their motility, but are sometimes immobile, so that the fact that the cultivated ones show no motility would not speak against the identity of the two spirilla.

As the cultures described, although isolated from healthy mouths, correspond so closely with those previously studied, which were obtained from cases of ulceromembranous angina and stomatitis, and diphtheria, it is probable that they are all identical. This is possible since apparently the same fusiform bacilli and spirilla occur in normal as well as in pathological conditions. There is good evidence that these organisms are the exciting agents of the various morbid processes with which they are associated on account of their presence in the lesions in large numbers and because of successful animal inoculations. From these facts and from the similarity between the cultivated and the uncultivated organisms, it would seem that the spirilla found in these lesions are also simply a stage in the growth of the fusiform bacillus, and that the bacilli and spirilla are not distinct organisms, as has been thought. Further study of these organisms will be necessary to settle this question indisputably. That spirilla or spirochetæ can develop from bacilli is now evident; whether they all do can be shown only by further investigation. If the spirilla found in the tissues are simply a later stage in the development of the fusiform bacillus, the fact may be explained why sometimes bacilli alone, spirilla alone, or a combination of the two occur. As a rule, if lesions are examined early, the bacilli only are found and later the spirilla. It has been observed that the cases in which the bacilli only occur are milder than when the two forms are associated. This may be accounted for by the fact that conditions are unfavorable for further growth of the bacillus so that the spirillary stage is not reached. It is stated that in cases in which deep destruction of tissue occurs, the spirilla are constantly present. This may be explained by the possibility that the length

of time required for the destruction of tissue and for the growth of spirilla may be the same.

It may be true that other bacilli, which up to this time have not been artificially cultivated, also formed spirilla in their growth.

The theory has been advanced by Wright¹ and Mackie² that fusiform bacilli and spirilla are forms of trypanosomes. From the study of these pure cultures, no evidence of their protozoan nature could be obtained.

I wish to thank Dr. Hektoen and Dr. Weaver for many suggestions.

¹*Lancet*, 1904, 2, p. 73.

²*Ibid.*, 1905, 2, p. 110.

EXPLANATION OF PLATE 2.

(Photomicrographs 1, 2, and 3 are of smears stained with carbol-gentian-violet, and 4 is of a smear stained with carbolfuchsin. $\times 1500$.)

FIG. 1.—Pure culture grown 48 hours anaerobically on Loeffler's blood-serum.

FIG. 2.—Pure culture grown 48 hours anaerobically in the fluid of condensation of Loeffler's blood-serum.

FIG. 3.—Pure culture grown four days in ascites broth.

FIG. 4.—Smear from gum in normal mouth.

FURTHER STUDIES ON STREPTOCOCCUS INFECTIONS.*†

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ABOUT two years ago Dr. Weaver and I¹ showed that the blood serum from scarlet-fever patients has no streptococcidal power *in vitro*, in any stage of the disease. Through the researches of Lemoine, Slawyk, Hektoen, and others we know that the streptococci are occasionally found circulating in the blood of these patients, and that the patients in whose blood the cocci are found often make an uneventful recovery. Jochmann,² however, is of the opinion that the prognosis is very bad when streptococci are found in the blood. What is true of scarlet fever may equally well be said of tonsillitis. Here, too, streptococci may invade the blood stream, but in spite of this fact the patient may make a rapid and satisfactory recovery.³ In erysipelas and in many wound and puerperal infections the tissues are invaded by virulent streptococci, but we know that in a great majority of these cases the organisms disappear sooner or later, and the patients make a complete recovery.

We know that normal human serum has no streptococcidal power. Hence the disappearance of cocci from the blood and tissues cannot be explained by assuming that the serum lacks something during the height of the disease, but regains this substance as convalescence sets in, and thus destroys the cocci. It is evident, therefore, that we must look for some other agent than the serum alone to account for the disappearance of the cocci from the blood and tissues during convalescence. According to Metchnikoff, Denys and Leclef, Bordet, Marchand, and others,⁴ this agent is found in the phagocytes. This view has not been universally accepted, although experiments show

*Received for publication January 10, 1906.

†This work was aided by a grant from the American Medical Association, and was published in the *Jour. A. M. A.*, 1906, 46, p. 108.

¹*Trans. Chicago Path. Soc.*, 1903, 5, p. 285. *Medicine*, 1903, 9, p. 515.

²*Deutsches Arch. f. klin. Med.*, 1903, 5, p. 209.

³ROSENOW, *Am. Jour. of Obstetrics*, 1904, 50, p. 766.

⁴For a more complete review of the literature on phagocytosis see *Jour. A. M. A.*, 1905, 44, p. 198.

that it explains the facts better than any other theory that has been advanced. In a previous paper¹ I was able to show that the leucocytes are the most important, if not the only, factor concerned in the destruction of streptococci in the body of infected rabbits and guinea-pigs. The view was there expressed that in man also the leucocytes may play an important rôle in combating infection by these organisms, although not much work had been done on human infections.

In this paper are set forth the results of further studies of streptococcus infections in human subjects, in the hope of determining just what factors are concerned in the destruction of the invading cocci; and an attempt is made to analyze more fully this phenomenon. In all instances where blood was used, it was drawn from the vein at the elbow by means of a Luer syringe, and defibrinated by gently whipping with a sterile wire. If the defibrinating is carefully performed, not a very large proportion of the leucocytes are destroyed. In the experiments each tube contained 0.8–1.0 c.c. of blood or serum, which was inoculated with one loopful of streptococcus culture, and two loopfuls from each tube were plated in glucose agar at intervals. The tubes were always kept in the incubator at 36°C. When highly virulent organisms were used, 0.3–0.4 c.c. of defibrinated rabbit blood was added to each tube of melted agar to facilitate the counting of colonies which are often very small if no blood has been added.

Table 1 shows that, although human serum, *in vitro*, is a good culture medium for streptococci, normal defibrinated blood has a slight streptococcidal power. Occasionally we may find a sample of normal blood which destroys many non-virulent streptococci, but the virulent organisms usually multiply in this blood. Table 2 shows that defibrinated blood from patients suffering from an acute infection has a much greater destructive effect upon these organisms than has normal blood. In these infections the leucocyte count is usually somewhat increased, and it seems that the streptococcidal power of the blood, *in vitro*, is roughly proportional to the leucocyte count. That is, the higher the leucocyte count, the greater will be the streptococcidal power of the blood. This is a general rule, to which there are, however, a few exceptions, to be pointed out later. The

¹*Jour. A. M. A.*, 1905, 44, p. 198.

virulent organisms frequently multiply in these bloods unless the leucocytosis is quite high. In no instance could a streptococcal power of the serum alone be detected. It might be objected that we are not dealing here with an actual destruction of cocci, but that the decrease in the number of colonies on the plates is due to adherence of the cocci to the leucocytes. This objection is ruled out by the fact that the 24 hour plates from tubes containing blood with a high leucocytosis are very often sterile, or nearly so.

It has been thought possible that the serum, during the course of an infection which terminates favorably, might acquire streptococcal properties for that particular race of streptococci which is responsible for the infection, while at the same time it had no such properties for other races of these organisms. Three strains of streptococcus were therefore isolated from erysipelas patients, and the serum of each patient tested on the corresponding organism. All of the patients made a satisfactory recovery, but at no time could streptococcal properties be demonstrated in their serum. The defibrinated blood, on the other hand, killed many cocci, while there was an increased leucocytosis.

We know that there is an intense local reaction in the localized streptococcus infections, and it has been thought by some that there may be lysis of cocci by the inflammatory serum in these areas. This

TABLE I.
THE EFFECT OF DEFIBRINATED NORMAL HUMAN BLOOD AND HUMAN SERUM UPON STREPTOCOCCI.

STREPTOCOCCI	DEFIBRINATED BLOOD	COLONIES ON AGAR PLATES		
		Immediate	2-3 Hours	5 Hours
300*	I	1,100	160
300	II	3,000	1,060
300	IIa	680	1,300
300	III	2,600	2,700	3,500
300	IV	3,000	1,050	2,600
300	V	540	300	240
300	VI	1,100	500	300
298	VII	1,600	600	315
298	VIII	600	80	16
B104	IIa	76	55	510
B104	III	1,800	2,800	Many
B104	IV	1,600	3,000	"
B104	V	500	600	900
381	VI	2,000	2,300	Many
300	Serum VI	1,400	2,000	"
298	Serum VII	1,700	2,500	10,000

* Streptococcus 300 and 298 are non-virulent, as they have been kept on artificial media for two years. B104 and 381 have been passed through many rabbits and are highly virulent.

theory is difficult to confirm or refute, on account of the difficulty of obtaining inflammatory serum in the same condition as that in which it is found in the tissues. As it is not uncommon to find blebs of considerable size on the affected parts of erysipelas patients, the fluid from these blebs was taken as the nearest approximation to the inflammatory serum. This blister fluid, from several cases of erysipelas, was tested for streptococcidal properties, but gave negative results.

TABLE 2.

THE EFFECT OF DEFIBRINATED BLOOD FROM CASES OF SCARLATINA, ERYSIPELAS, TONSILLITIS, AND PNEUMONIA UPON STREPTOCOCCI.

STREPTOCOCCI	DEFIBRINATED BLOOD	COLONIES IN GLUCOSE AGAR PLATES			
		Leucocyte Count	Immediate	2-3 Hours	5 Hours
300	Scarlatina I	10,500	1,100	6
300	" II	10,600	2,000	140
300	" III	15,000	690	116
300	" IV	550	3
300	" V	1,500	300	150
300	" VI	1,900	350	43
300	" VII	500	12
300	" VIII	13,000	1,380	102	9
300	" IX	1,200	125	14
300	" X	13,400	1,250	245	120
300	" IX	1,950	150	19
300	" XII	10,800	1,800	700	130
300	" XIII	1,800	300	45
300	" XIV	12,300	1,500	31	2
381	" III	15,000	1,200	1,500	3,500
B104	" IV	600	462	415
B104	" V	1,800	1,800	5,000
381	" VI	220	51
381	" VII	130	41	1,100
381	" VIII	13,000	360	1,180	Many
381	" IX	13,000	2,200	1,950	"
B104	" IX	330	190	1,500
381	" X	13,400	650	290	2,500
B104	" X	400	160	165
381	" X	320	650	Many
381	" XII	10,800	200	2,000	"
381	" XIII	540	900	"
300	Erysipelas I	630	64	0
300	" II	15,000	800	14	4
300	" IIa	1,100	41	8
300	" III	740	420	160
300	" IV	1,650	480	150
298	" V	10,400	3,100	420	92
B104	" I	1,200	1,080	960
B104	" II	96	30	13
B104	" IV	133	110	480
300	Tonsillitis I	16,000	620	34	2
B104	" I	300	59	70
300	" II	1,400	74	6
300	Pneumonia I	2,600	0	3
300	" II	00	5	0
300	Ser. scar. VII	1,500	3,500	10,000
300	" XIV	1,200	1,400	1,300
300	Erysipelas II	670	760	750
300	" III	700	1,800	6,000
300	" V	3,000	3,500	5,000
B104	" V	190	360	1,140

The importance of a high leucocyte count in the destruction of streptococci by blood is clearly shown by the accompanying record of experiments.

Experiment 1.—Ten c.c. of blood were drawn from the vein at the elbow of an erysipelas patient, and carefully defibrinated. One c.c. of the defibrinated blood, which contained 9,800 white corpuscles per cubic millimeter, was put into small test tubes, inoculated with one loopful of virulent streptococcus culture, and two loopfuls of the inoculated blood plated at intervals. The remaining 8 c.c. were centrifugated and most of the serum was drawn off. We know that the uppermost stratum of centrifugated corpuscles contains a high percentage of leucocytes, because they are thrown down less easily than the red corpuscles. This stratum was therefore drawn off with a sterile pipette, and mixed with a small quantity of serum. The resultant mixture contained 17,200 leucocytes per cubic millimeter. One c.c. of this "suspension of leucocytes" was introduced in a small test tube, inoculated, and plates were made as before. To complete the experiment, 1 c.c. of the clear serum was put into a small tube, which was likewise inoculated, and plates were made at intervals. This experiment was also performed with normal blood and a non-virulent streptococcus. The plates were incubated for 24 hours, the colonies that developed on each were counted, with the results shown in Table 3.

TABLE 3.

STREPTOCOCCUS		LEUCOCYTE COUNT	COLONIES ON AGAR PLATES		
			Immediate	2-3 Hours	5 Hours
B104	Erysipelas blood	9,800	390	168	350
B104	Suspension erysipelas leucocytes	17,000	260	58	21
B104	Erysipelas serum	200	360	1,100
298	Normal blood	4,500	1,650	600	316
298	Suspension normal leucocytes	6,600	1,600	420	62
298	Normal serum	1,700	2,500	00

The table shows that both strains of streptococcus which were used multiplied in the cell-free serum; that the defibrinated blood destroyed many of the non-virulent and some of the virulent cocci; and that the "suspension of leucocytes" destroyed more cocci of either strain than did the defibrinated blood. The only difference between the defibrinated blood and the suspension of the leucocytes lay in the fact that the latter contained nearly twice as many leucocytes as the former.

The fact that the streptococcidal power of the blood is dependent upon its number of leucocytes per cubic millimeter has also been demonstrated by a second experiment.

Experiment 2.—Shortly after a patient's admission into the hospital 3 c.c. of blood were drawn from a vein at the elbow and defibrinated. The leucocyte count at this time was 11,000. The blood was divided equally among three tubes, each tube was inoculated with streptococcus culture, and two loopfuls from each were plated at intervals. Shortly after drawing the blood, the patient was injected under the skin of the back with 10 c.c. of an antistreptococcus serum. This brought about an increase in the leucocytosis up to 15,000* five hours after the injection. Three c. c. of blood were again drawn from the vein at the elbow, and its effect on streptococci was tested as before. The results of the experiment are shown in Table 4.

TABLE 4.

STREPTOCOCCUS		LEUCOCYTOSIS	COLONIES ON AGAR PLATES		
			Immediate	2-3 Hours	5 Hours
300.....	Blood before injection	11,000	1,100	360	200
B104.....	" " "	390	380	4,000
381.....	" " "	100	500	Many
300.....	Blood 5 hrs. after injection	1,500	1,300	44	0
B104.....	" 5 " " "	412	246	600
381.....	" 5 " " "	150	270	3,000

The blood drawn after the injection of the serum, when the leucocytosis was high, has a greater streptococcidal power than that drawn before the injection. It would not be safe to conclude that this difference in the streptococcidal powers is due entirely to the difference in the leucocyte count. Some of it might be due to an antitoxic or opsonic action of the injected serum, or to a stimulation of the leucocytes. This supposition loses most of its force when we consider the fact that the addition of 1 to 5 per cent of antistreptococcus serum to defibrinated blood, *in vitro*, does not increase its streptococcidal power, as shown by Dr. Hektoen and myself.

Wright and Douglas¹ have shown that phagocytosis takes place only after the bacteria have been sensitized, that is, have been acted upon by the opsonin of the serum. There is no phagocytosis of a suspension of washed leucocytes in NaCl solution, or in heated serum, regardless of the number of untreated bacteria that are added. This work has been confirmed and extended by Hektoen and Ruediger,² and by Bulloch and Atkin.³ In view of these facts, there should be no reduction in the number of streptococci in a test tube containing

* I am indebted to Dr. Tunncliff for the leucocyte counts and the injection of the serum.

¹ *Proc. Roy. Soc.*, 1903, 72, p. 357, and 1904, 73, p. 128.

² *Jour. Infect. Dis.*, 1905, 2, p. 128.

³ *Proc. Roy. Soc.*, 1905, 74, p. 379.

a suspension of washed corpuscles in salt solution or in heated serum, and the following experiment shows that such is the case.

Experiment 3.—Ten c.c. of blood were drawn from a vein at the elbow of a scarlet-fever patient, defibrinated, centrifugated, and the serum drawn off. The corpuscles were washed twice in a large amount of NaCl solution and 0.5 c.c. of the centrifugated corpuscles placed into each of three small tubes containing 0.5 c.c. of normal serum, 0.5 c.c. of heated (58° for one-half hour), and 0.5 c.c. of salt solution, respectively. The tubes were inoculated with one loopful of streptococcus culture, and two loopfuls from each were plated at intervals, with the results shown in Table 5:

TABLE 5.

STREPTOCOCCUS		COLONIES ON GLUCOSE AGAR PLATES		
		Immediate	5 Hours	24 Hours
300.....	Washed corpuscles + serum	1,500	7	21
300.....	" " + heated serum	1,400	5,000	Many
300.....	" " + NaCl solution	1,700	3,500	"
300.....	Serum	1,200	1,400	

The importance of opsonin in the destruction of streptococci is further shown by the fact that the defibrinated blood from two obstinate cases of post-scarlatinal nephritis had no streptococcidal powers, although in one of these cases the leucocyte count was 14,000. When the blood from these patients was centrifugated, and the corpuscles suspended in serum from a patient who was convalescent and had no nephritis, the resultant suspension had a small degree of streptococcidal power, as shown by Table 6:

TABLE 6.

STREPTOCOCCUS		COLONIES ON AGAR PLATES		
		Immed.	2 Hours	5 Hours
298.....	" Convalescent blood "	800	450	14
298.....	" Nephritis blood "	720	1,900	6,000
298.....	" Nephritis corpuscles " + " conv. ser. "	850	500	540
298.....	" Conv. washed corp. " + " neph. ser. "	725	600	61

The interesting fact came to light in these experiments that the combination "convalescent washed corpuscles" + "nephritis serum" has nearly as great a streptococcidal power as the defibrinated blood from the patients without nephritis. The corpuscles were washed twice in a large amount of NaCl solution, which is usually

sufficient to prevent phagocytosis in a suspension of corpuscles in NaCl solution. It is not likely, therefore, that the washing had not been carried far enough. But the results of these experiments seem to indicate rather that the leucocytes, as well as the serum, from these nephritis patients have undergone some change which renders them less efficient in the destruction of bacteria. In fact, it would seem that the leucocytes have suffered more than the serum. Whether or not these facts may serve to throw light on the cause of some of the terminal infections cannot be determined at this time.

Whether or not the opsonin is increased during the acute infections is an interesting question. Normal leucocytes in normal serum take up large numbers of cocci; hence it is difficult to determine if leucocytes in erysipelas serum, for instance, take up more cocci than those in normal serum. This question had, therefore, to be approached in a different way. Two sets of tubes were made, and 0.2 c.c. of washed corpuscles introduced into each. To one set of tubes were added falling quantities of normal serum, and to the other set falling quantities of erysipelas serum. The contents of each tube were made up to 0.4 c.c. with NaCl solution, and to each tube was added 0.4 c.c. of a suspension of streptococci. The tubes were incubated for one hour at 36° C., smears made, and the average number of cocci in each leucocyte determined by counting those in 30 leucocytes. The results are shown in Table 7:

TABLE 7.

Normal Serum	Phagocytosis	Erysipelas Serum	Phagocytosis
0.2	13.5	0.2	11.5
0.1	9.4	0.1	11.2
0.05	6.7	0.05	8.2
0.025	4.7	0.025	6.0
0.012	2.2	0.012	4.3

This experiment indicates that there is a slight increase of opsonin in the erysipelas serum as compared with normal serum. A similar increase of opsonin has been noted by Wright and Douglas¹ after treating a person afflicted with furunculosis with their staphylococcus vaccine. A diminution of opsonin in persons subject to

¹ *Proc. Roy. Soc.*, 1904, 73, p. 128.

attacks of furunculosis, sycosis, etc., has also been observed by these investigators.

CONCLUSIONS.

Human serum does not acquire streptococcidal properties during the course of a streptococcus infection.

The blister fluid from erysipelas patients has no streptococcidal powers.

Defibrinated human blood has a streptococcidal power, which, with few exceptions, is roughly proportional to the leucocytosis.

The destruction of cocci in the defibrinated blood is brought about by the leucocytes, but, before this can be accomplished, the cocci must be acted on by the opsonin of the serum. There is no phagocytosis, and hence no destruction of unsensitized cocci by washed leucocytes.

The opsonin is increased during the course of an attack of erysipelas.

In an attack of acute nephritis the opsonin is diminished, but the leucocytes also undergo a change which renders them less effective in the destruction of streptococci. This fact may serve to throw light on the cause of many terminal infections.

I wish to thank Professor Hektoen for many suggestions, and the internes at Cook County Hospital for many courtesies.

THE ETIOLOGY OF PEMPHIGUS CONTAGIOSUS IN THE TROPICS.*

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DERMATOLOGISTS will no doubt continue to disagree upon the classification of the various forms of Pemphigus, until this has been placed upon a satisfactory etiological basis. Perhaps the following classification, modified from that of Peter,¹ is as satisfactory as any:

Pemphigus	{	1. Chronic forms, proper pemphigus (Hebra), Pemphigus vulgaris and foliaceus.		
		2. Acute febrile pemphigus.		
		<table> <tr> <td>Pemphigus</td> <td>{ "Pemphigus neonatorum."</td> </tr> <tr> <td>contagiosus</td> <td>{ "Impetigo contagiosa" (?)</td> </tr> </table>	Pemphigus	{ "Pemphigus neonatorum."
Pemphigus	{ "Pemphigus neonatorum."			
contagiosus	{ "Impetigo contagiosa" (?)			

Concerning the etiology of the diseases in the first group nothing is known.

We shall discuss the second group but briefly, and only in relation to the cases we have to report, without attempting to give a complete critical review of the literature upon the subject.

Acute febrile pemphigus may be considered a specific infectious disease, due to the diplococcus first described by Demme.² His observations have been confirmed by a number of workers, among them Bleibtreu,³ Pernet,⁴ Pernet and Bulloch,⁵ Whipham and Wells,⁶ Hadley and Bulloch,⁷ Hamburger and Kubel,⁸ and Eustis.⁹ The frequency of its occurrence in butchers, and its possible origin from lower animals are discussed by Pernet. Its apparent relation to foot and mouth disease of cattle, as deduced by Bowen,⁹ is rather far-fetched in view of the work of Löffler and Frosch on the latter

* Received for publication December 1, 1905.

¹ *Berl. klin. Wchnschr.*, 1896, 33, p. 124.

² *Verhandlungen des Congresses für Innere Medicin*, Wiesbaden, 1886.

³ *Berl. klin. Wchnschr.*, 1893, 30, p. 671.

⁷ *Ibid.*, 1899, 1, p. 1219.

⁴ *Brit. Med. Jour.*, 1895, 2, p. 1554.

⁸ *Johns Hopkins Hosp. Bull.*, 1903, 4, p. 63.

⁵ *Brit. Jour. Dermatology*, 1896, 8, p. 157.

⁹ *Amer. Med.*, 1904, 7, p. 634.

⁶ *Lancet*, 1896, 1, p. 1220.

¹⁰ *Jour. Cutaneous Dis.*, 1904, 22, p. 253.

disease (1897). The fact that the diplococcus of Demme is nonchromogenic, retaining its diplococcus form and arrangement, or appearing in short chains, in ordinary broth cultures, is sufficient to distinguish it from the following diplococcus.

Almquist¹ isolated from a series of cases of Pemphigus neonatorum a diplococcus which he named *M. pemphigi neonatorum*. This organism appears as diplococci ($0.5\ \mu$) in the contents of the blisters. Upon artificial media it closely resembled a control culture of *Staphylococcus pyogenes aureus* isolated from a carbuncle, growing like a yellow streak of paint on agar, liquefying gelatin, and producing turbidity with a yellow deposit in broth. It grew well at 20°C ., but poorly at 15°C . The pigment production varied, as it often does in the case of chromogenic microorganisms.

Using cultures grown for about 20 days upon artificial media, Almquist performed inoculation experiments upon himself, under conditions which leave no doubt that he was dealing with the etiological factor. Typical blisters, 1 cm. in diameter, or slightly larger, were produced, and the microorganism recovered in cultures. Almquist points out that, although his culture resembles that of the yellow pus coccus, it differs essentially in its action upon the human tissues, the process always remaining superficial, and resolution taking place without scarring. The superficial and non-febrile nature of the disease militates against the idea of a blood infection. One examination proved negative. Cultures dried on silk threads were viable after one and a half months. He concluded that the tenderness of the new-born makes it more susceptible, and that the contagion is transferred mechanically from one person to another.

There is considerable evidence in support of the statement made by Kunt Faber, in 1890, to the effect that Pemphigus neonatorum and Impetigo contagiosa should be classed as identical diseases, under the common name of Pemphigus contagiosus. Matzenauer² made a histological and bacteriological study of both Pemphigus neonatorum and Impetigo contagiosa, and concluded that they were identical. However, he considered that the organisms which he isolated were staphylococci, indistinguishable from the *Staphylococcus pyo-*

¹ *Ztschr. f. Hyg.*, 1891, 10, p. 253.

² *Virchow-Hirsch, Jahrb. d. ges. Med.*, 1900, 2, p. 549.

genes aureus. Leiner¹ studied cases of Pemphigus contagiosus in association with measles. He was successful in producing Impetigo contagiosa in an individual inoculated from the blisters of a case; and since organisms which he considered to be staphylococci were isolated from both diseases, he concluded that they were identical.

Pemphigus contagiosus has a wide geographical distribution, and occurs in many parts of the tropics—"wherever heat and moisture combine to bring about a state of the skin favoring its development on the infective material being applied." "European children are more prone to it than native children; European adults are by no means exempt, but the native adult is rarely affected."² Many years ago Manson³ noted the presence of a diplococcus in the contents of the blisters, but performed no inoculation nor cultivation experiments.

In 1904 we made bacteriological examinations on five cases of Pemphigus neonatorum occurring in the Civil Hospital, Manila, P. I. Practically every child born in the maternity ward of this hospital contracts the disease during the period of the mother's convalescence. Only the lack of time prevented us from utilizing the abundant material. From all five cases studied by us the microorganism described by Almquist was isolated. It occurred as distinct kidney-shaped diplococci in the contents of the blisters, and appeared quite like *Staphylococcus pyogenes aureus* on culture media, yet showed some features which we think distinctive. We shall not describe these cultures here, as they are identical with the diplococcus described below from a case of Pemphigus contagiosus in an adult.

CASE HISTORY.

June, 1904, Mr. G., American schoolmaster, about 40 years old, stout and in good health, complained of an eruption in both axillæ. He had been troubled with much itching and scratching owing to "prickly heat." About four days ago he noticed a few vesicles forming in the axillæ. These increased in number, and later some appeared on the back of his neck, on the scalp and on the legs.

At present both axillæ are covered with a maculo-papular and

¹ *Jahrb. f. Kinderh.*, 1902, 55, p. 316.

² MANSON, *Tropical Diseases*, 1900, p. 611.

³ *Hong Kong Medical Reports*—reference not accessible.

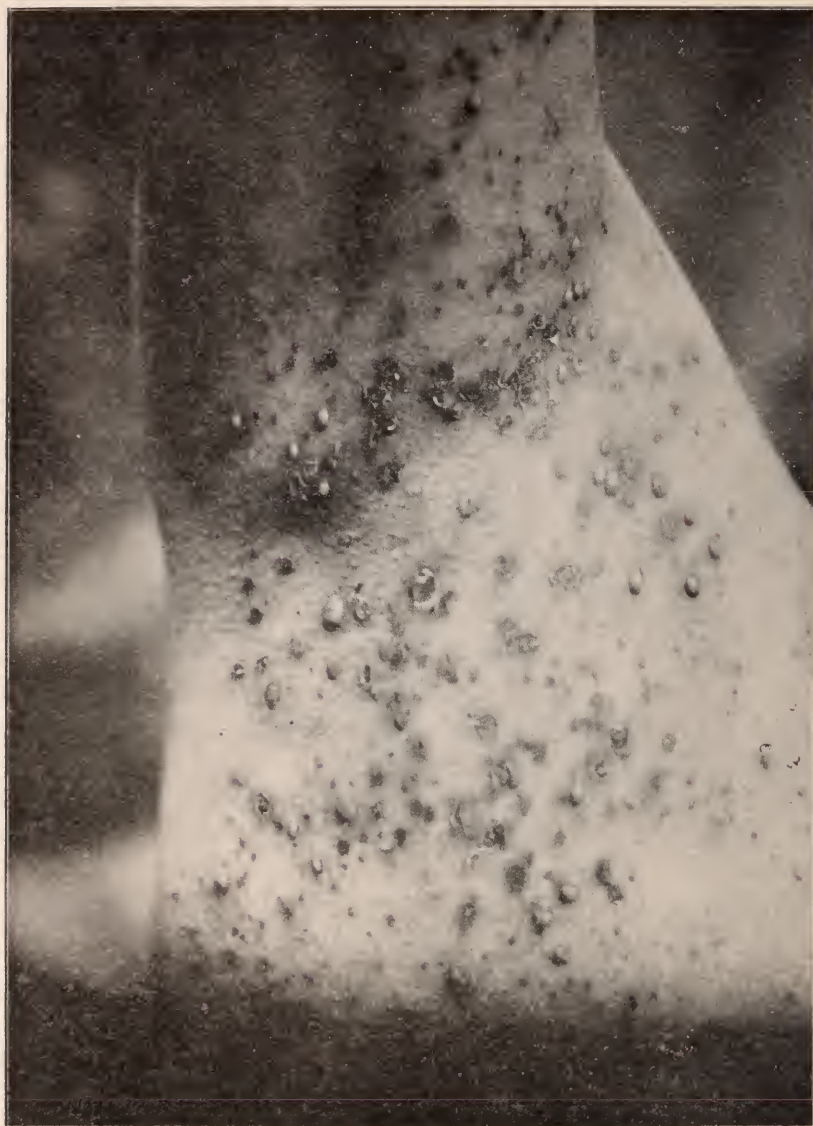


FIG. 1.

vesicular eruption (Fig. 1). The vesicles, at first minute, rapidly increase to 5 or 6 mm. in diameter, while their contents become purulent. Some are surrounded by an aureola of hyperemia, others

not. On rupture they leave a red, shining, inflamed base. Where the eruption is thickly set, the intervening skin shows a scarlatini-form blush, but this appearance may be due to the minute maculopapular eruption of "prickly heat." The eruption is accompanied by sensations of stinging and itching, and the muscles of the parts feel sore upon pressure. Two of his children had a similar eruption about two months ago. After a few days' treatment with bichloride of mercury washes, followed by a mild dusting powder, the eruption disappeared.

BACTERIOLOGICAL EXAMINATION.

Coverslip preparations from the contents of the vesicles showed an enormous number of kidney-shaped diplococci which apparently were present in pure culture. Many of the organisms were inside of polynuclear leucocytes. In fact, a preparation closely resembled one from gonorrheal pus (Fig. 2.). Compresses of bichloride of mercury (1:1,000) were applied, and the surface washed thoroughly with sterile salt solution. Seven successive stroke cultures were made upon + 1 agar

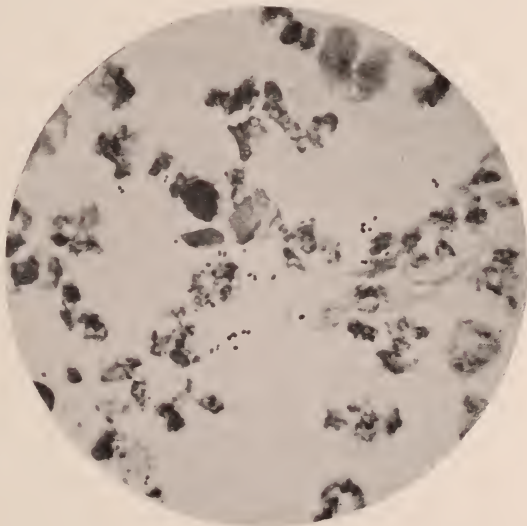


FIG. 2.

slants¹ from vesicles from the right axilla, three from a vesicle in the left, kept at 36-37° C. In 24 hours all the tube were covered with numerous discrete, golden-yellow colonies varying from pin-point size to 0.5 mm. in diameter. On the slants which received the first inoculation the colonies were so numerous that they soon became confluent. Pure cultures from isolated colonies on each set were studied.

¹The media were prepared according to the recommendations of the American committee, excepting that the reaction to phenolphthalein was adjusted by the addition of sodium hydroxide alone. *Amer. Pub. Health Assoc. Rep.*, 1898, 23 p.60.

Biochemical characters.—Upon the ordinary media growth appeared like that of a culture of *Staphylococcus pyogenes aureus* isolated from a case of appendiceal abscess. Litmus milk was coagulated in about a week, and gelatin (final reaction +2) liquefied. No indol could be demonstrated in +1 or -1 broth, nor cholera red in Dunham's peptone solution containing 0.01 per cent KNO_3 , after 10 days' growth at 35-37°. In +1 glucose broth containing one-third part of sterile goat serum, growth appeared with remarkable rapidity, a tube being densely clouded, while control tubes inoculated with *Staphylococcus pyogenes aureus* and *Sarcina lutea* showed only a faint cloudiness. With the formation of acid the serum was precipitated as a dense flocculent mass.

In +1 broth containing 1 per cent of glucose, lactose, and saccharose in the fermentation tube, growth appeared in the closed branch as well as in the bulb, but no gas was produced. No distinct diplococci were seen in preparations from the open and closed branches.

The morphology in preparations from agar and broth cultures was practically indistinguishable from that of pyogenic staphylococci, but when made from milk, or better, serum broth, the diplococcus arrangement found in smears from the vesicle contents was well reproduced. As in the case of many chromogenic bacteria, better pigment production occurred on glycerin and glucose than on plain agar.

Pathogenicity.—Twenty days after inoculation, 1 c.c. of a 48 hour-old serum broth culture was injected into the peritoneal cavity of a 484 gm. guinea-pig. It remained well for a week. Discarded.

A rabbit's back was shaved, the skin sterilized, and small amounts of the above serum broth culture injected subcutaneously. No vesicles resulted, and only small hyperemic areas appeared. These disappeared in a week.

Seven days after isolation (third or fourth subculture), one of us inoculated himself on the forearm. An area on the anterior aspect of the forearm was sterilized with alcohol and bichloride of mercury (1:1,000), followed by thorough washing with sterile salt solution. The point of a needle was dipped into a recent serum broth culture of the diplococcus, and a number of slight pricks were made through the skin. The area was then protected with an aseptic vaccination

shield. In four hours small areas of congestion appeared. In 20 hours these had enlarged. The two largest were about 3 mm. in diameter, and showed minute vesicles about 1 mm. in diameter. In 30 hours these two cloudy vesicles had enlarged to about 2 mm. in diameter, and two more 1 mm. vesicles had appeared. A slight sensation of itching was noticeable. In 48 hours, however, the eruption underwent resolution.

SUMMARY AND CONCLUSIONS.

1. From five cases of "Pemphigus neonatorum" and one case of Pemphigus contagiosus in an adult, micrococci similar to those described by Almquist were isolated.

2. Although occurring as well-defined kidney-shaped diplococci in the contents of the vesicles, the organism may, on superficial examination of cultures, be confounded with *Staphylococcus pyogenes aureus*. Our cultures did not produce indol in broth, and the diplococcus arrangement was reproduced in milk, or, better, in serum broth cultures.

3. A single human inoculation experiment with this organism produced typical but abortive vesicles. The essentially superficial nature of the inflammatory process set up in the human skin—resulting in the exudation of serum and leucocytes, and the formation of vesicles and the absence of any tendency to penetrate into the deeper tissues, certainly differentiate this micrococcus from the ordinary pyogenic cocci.

4. We believe it advisable to call the disease *Pemphigus contagiosus*, whether occurring in children or adults, and the etiological factor would then best be termed *Micrococcus pemphigi contagiosi*.

5. Cases of typical "Impetigo contagiosa" should be examined along similar lines, as the disease described under this name is possibly due to the same microorganism.

The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 3

April 6, 1906

No. 2

THE SIGNIFICANCE OF STREPTOCOCCI IN MILK.*

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OF late years attention has been frequently called to the presence streptococci in milk. Von Lingelsheim¹ mentions the fact that streptococci are found in market milk, and suggests that these bacteria may be the cause of many cases of infantile intestinal diseases. Streptococci in milk have also been reported by Conn,² Conn and Esten,³ Bergey,⁴ Barthel,⁵ Reed and Ward,⁶ who also found them in the udder, and others, and special stress has been laid on the significance of leucocytes in market milk if streptococci are present. An examination by the writer of fresh milk of the highest character, in the summer of 1905 resulted in finding that streptococci are invariably present even in milk obtained with unusual precautions and from healthy cows.

This led to an investigation of the relation between the streptococci and the lactic acid bacteria ordinarily concerned in the souring of milk, it being known, among other things, that streptococci can produce and withstand a large amount of acid.

Pasteur⁷ was the first to describe lactic acid bacteria. His "levure

*Received for publication, January 5, 1906.

¹KOLLE UND WASSERMANN, *Handbuch der pathogenen Mikroorganismen*, 1903, 3, p. 337.

²*Centralbl. f. Bakt.*, Abth. 2, 1902, 8, p. 442.

³*Ann. Report Storrs Agric. Exp. Station*, 1902-3, p. 63.

⁴*Dept. of Agriculture, Commonwealth of Penna.*, Bull. 125, 1904, p. —.

⁵*Milchzeitung*, Leipzig, 1903, pp. 40, 41, 42.

⁶*Centralbl. f. Bakt.*, Abth. 1, 1903, 30, p. 83.

⁷*Ann. de chim. et phys.*, Série 3, 1858, 52, p. 404.

lactique" consisted of "small globules or very short particles resembling certain kinds of amorphous precipitates. They are constricted in the middle, occur isolated or in irregular clusters. They ferment lactose with acid and gas formation, and cultures are often viscid." In 1878 Lister¹ isolated an organism from sour milk by the dilution method which he called *Bacterium lactis*, a detailed description of which could not be found. In 1884 Hueppe² described his *B. acidi lactici*, which he found in large numbers in all samples of milk examined, and which was subsequently accepted as the lactic acid organism *par excellence*. This organism so far as can be gathered from the original description, is to be considered as a variety of *B. [lactis] aërogenes*, Escherich. Following this discovery many varieties of lactic acid bacteria have been described, some of which may be identified with the one discovered by Hueppe. Clauss,³ Grotenfelt,⁴ Schardinger,⁵ Kozai(2)⁶ and Utz (2),⁷ have isolated lactic acid bacteria which apparently are identical with, or at most varieties of, *B. aërogenes*. The luxuriant growth on all media is characteristic. On agar, in ordinary broth, on potato, and in proteid-free media, there is abundant growth. Dextrose and lactose are fermented with acid and gas formation; milk is coagulated more or less rapidly, the coagulum contracts characteristically and whey is separated. Colonies on agar or gelatin are large, moist, and often viscid. Stab cultures produce good growth on the surface and in the puncture.

A wholly different class of lactic acid bacteria has been described by Leichmann,⁸ Günther and Thierfelder,⁹ Kozai (1),¹⁰ Utz (1),¹¹ Esten,¹² Schierbeck,¹³ and others. Günther and Thierfelder identify their organism erroneously with Hueppe's. These bacteria have been usually described as bacilli, but regarded as distinct from the *B. aërogenes* type. Leichmann was the first to call attention to this difference and unfortunately named his organism *B. lactis acidi* in distinction from *B. acidi lactici*, Hueppe. This appellation has been the cause of great confusion.

¹Quarterly Jour. of Microscopical Science, 1878, 18, p. 177.

²Mitth. a. d. kais. Gesundh. Amt., 1884, 2, p. 309. ⁸Ibid., 1896, 2, p. 777.

³Inaug. Dissert., Würzburg, 1889.

⁹Arch. f. Hyg., 1895, 15, p. 164.

⁴MIGULA, System der Bakterien, 1900, 2, p. 544.

¹⁰Loc. cit.

⁵Monatshefte f. Chemie, 1890, 11, p. 544.

¹¹Loc. cit.

⁶Ztsch. f. Hyg., 1899, 31, p. 337.

¹²Ann. Rep. Storrs Agric. Exp. Station, 1896.

⁷Centralbl. f. Bakt., Abth. 2, 1904, 11, pp. 600 and 733. ¹³Arch. f. Hyg., 1901, 38, p. 294.

The experiments reported in this paper comprise first a comparative study of cultural characteristics of the following organisms:

Four lactic acid bacilli, true bacilli, one of which was in the laboratory collection labeled *B. acidi lactici*; the other three were isolated from loppered milk. These all proved to be of the *B. aërogenes* type.

Eight lactic acid organisms received from the following laboratories under the name *B. acidi lactici*: One from Professor Russell, University of Wisconsin; three strains from Professor Harding, N. Y. Agric. Exp. Sta., Geneva, N. Y.; one from Professor Prescott, Mass. Institute of Technology, Boston, Mass.; one from Professor Marshall, Michigan Agricultural College; one from Professor Bouska, Ames Agricultural College, Iowa; and one from Professor Conn, Wesleyan University, Middletown, Conn. All of these were found to belong to the group *B. lactis acidi*, Leichmann.

In addition a streptococcus from Chicago milk, isolated in this laboratory by Dr. Harris in February, 1904, was obtained for this investigation in May, 1905, and a streptococcus isolated from sewage also by Dr. Harris. Five pathogenic streptococci isolated from cases of scarlet fever, tonsilitis, and phlegmon, by Dr. Weaver, Memorial Institute of Infectious Diseases, Chicago, in February, 1904, were received for this investigation in May, 1905.* Streptococci were then isolated by the writer from fresh milk, sour milk, separator slime, market milk, cow feces, and from drippings from the udder during the process of washing previous to milking. These latter agreed well with each other and also with the so-called *B. lactis acidi*. The experiments were carried on in duplicate, one set being kept at 37° C., the other at room temperature.† Except for the fact that growth, in the majority of cases, was somewhat better at 37° C. than at room temperature, no noteworthy difference was brought out by these different temperatures.

The bacilli of the *B. aërogenes* type are all negative to Gram's stain, are generally plump, oval, and in irregular clusters. Diplobacillus forms are not infrequent. Two have capsules, one is viscid

*I wish to acknowledge my gratitude to those who have kindly supplied me with cultures for this work.

†Media were prepared according to the methods prescribed by the Bacteriological Committee of the Amer. Pub. Health Assoc. (*Jour. Infect. Dis.*, 1905, Supplm. No. 1) and 2 per cent lactose added to agar, broth, and gelatin. The formula recommended by Jordan (*Botan. Gazette*, 1899, 27, p. 1) was employed for non-proteid media. Bacteria were isolated from milk by the usual plate method with lactose-litmus agar.

in cultures, and gas is formed by all from dextrose and lactose. On agar and potato and in gelatin and non-proteid media, with and without lactose, growth is luxuriant. The colonies are well developed on the surface, are large, moist, and viscid. No difference from typical *B. aërogenes* could be noted.*

A striking similarity was found to exist between bacteria of the streptococcus and the *B. lactis acidi*, Leichmann types. The characteristics of these are briefly as follows:

Morphology.—Cocci, sometimes oval, mostly in pairs, and often with pointed ends, frequently in chains of three to six members, and sometimes in chains of considerable length. Cultures in lactose broth showed chain formation to best advantage. The cocci are positive to Gram's stain.

Culture media.—Growth on slant agar and potato and in plain broth is uniformly poor, considerably better if lactose is added to media. On slant agar a delicate ribbon-like layer is formed, which often consists of single or confluent colonies, and in a few cases is hardly visible. On the surface of stab cultures there is a barely visible growth or none at all; in the puncture, however, it is quite fair. Coagulation of milk varies, from one to fourteen days being required. Litmus, if added to milk, is decolorized by *B. lactis acidi*, and all streptococci in the same typical manner. The solid coagulum turns white leaving a pink ring at the top, which gradually extends toward the bottom, and little or no whey is liberated. In non-proteid media there is no noticeable growth.

Colonies.—Small, irregular, appear mostly in the depths, but few on the surface.

The next inquiry concerned the variability of coagulative power. Experiments were conducted by passing each of the above-mentioned organisms, 33 in all, through eight tubes of litmus milk, successively. The milk employed was mixed previous to tubing and sterilizing, to insure uniform composition. Transfers were made every other day. The majority of streptococci, especially pathogenic ones, gained in coagulative power. The same was observed in streptococci from cow feces and sewage. The appearance of the coagulum of all streptococci and *B. lactis acidi*, Leichmann type was alike—showing the typical decolorization and the red ring on top.

The opinion of Houston,¹ that streptococci in water are of value in detecting sewage contamination, has received some support.² The

*The rules of nomenclature possibly demand that "*B. acidi lactis*, Hueppe" be adopted in place of *B. lactis aërogenes* on account of priority. As this would undoubtedly lead to confusion it seemed best for the present to retain the name "*B. aërogenes*."

¹ *Supplm. to the 29th Annual Rep. of the Local Gov. Board, containing the Rep. of Med. Officer for 1899-1900*, p. 458.

² WINSLOW AND HUNNEWELL, *Jour. Med. Res.*, 1902, 3, p. 502; PRESCOTT AND BAKER, *Jour. Infect. Dis.*, 1904, 1, p. 193.

method employed is to inoculate dextrose or lactose broth with definite amounts of the water in question. Bacteria of the Colon-Aërogenes group multiply rapidly, and when a certain amount of acidity is reached, streptococci, being able to resist a higher degree of acidity, crowd other bacteria out. Judging from analogy it is reasonable to assume that similar conditions obtain in milk. Three lines of research were carried on to establish this view.

(1) One quart of certified milk was divided into two parts and placed in two flasks. One of these was kept at 37° C., the other at room temperature. Dilutions were prepared daily for eight consecutive days and 1 c.c. of each dilution inoculated into each of five lactose broth tubes. These were examined after three days, and streptococci appeared in all instances in high dilutions. By this method the presence of streptococci even when they occur in very small numbers can be readily demonstrated. Dilutions prepared from fresh milk showed streptococci in chains of immense length. Litmus milk inoculated from these tubes coagulated in typical fashion in 24 to 48 hours, but the long chains had disappeared, and in their stead, diplococci and short chains of three to six members appeared. As the acid fermentation progressed, the long chain formation became more and more scarce, giving way to diplococcus and short chain formation. It appears from this experiment that long chains disappear if these lactic streptococci are cultivated in milk. (See plates.)

(2) Plates in lactose litmus agar were prepared for eight successive days from two flasks of milk from the same lot, undergoing spontaneous fermentation, one at 37° C. the other at room temperature. An approximate enumeration of the colonies of *B. aërogenes* and "*B. lactis acidi*," shows that at initial stages of fermentation *B. aërogenes* multiplies more rapidly than the other type; at later stages, however, the Leichmann type gains ascendancy. This phenomenon is more pronounced at room temperature than at 37° C.

(3) A third series of experiments was of the following nature: Ten liters of milk were mixed and evenly distributed in 42 flasks. Thirty-eight of these were sterilized in the Arnold for three successive days, and allowed to stand at room temperature for six days, before being used. The other four flasks were allowed to lopper, two at 37° C., the other two at room temperature. One of the sterilized flasks was

500 C.C. FLASKS OF STERILE MILK INOCULATED WITH PURE CULTURES, VARIOUS MIXTURES OF LACTIC ACID BACTERIA, SOUR MILK AND FLASKS WITH NATURALLY SOURED MILK.

NUMBER OF FLASK	INOCULATED WITH	ODOR	COAGULATION			ACIDITY PER CENT IN LACTIC ACID		Number of Days after Which Highest Acidity Was Reached
			In Days	Homogeneous	Broken up and Whey	At Time When Coagulation Was First Observed	Highest Acidity Reached	
1....	Control						0.164	1
2....	Pathogenic streptococcus	Sour mild	5	+	—	0.507	0.726	11
3....	" "	" "	5	+	—	0.508	0.773	11
4....	Lactic acid bacterium from sweet milk	" "	3	+	—	0.664	1.024	13
5....	Lactic ac. bact. from separator slime	" "	1	+	—	0.716	1.040	5
6....	Bacillus from sweet milk	Sour and Cheesy	3	—	+	0.541	1.006	11
7....	Bacillus from sour milk	" "	3	—	+	0.619	0.816	8
8....	B. acidi lactici, University of Chicago	" "	4	—	+	0.669	1.149	10
9....	1 c.c. sour old milk for inoculation	" "	1	—	+	0.707	1.130	6
10....	Lactic acid bacter. from N. Y. Station	Sour mild	4	+	—	0.532	1.056	9
11....	Lactic acid bacterium from teat wash water	" "	1	+	—	0.557	0.969	5
12....	Streptococcus from cow feces	" "	7	+	—	0.576	0.790	13
13....	Liquefying streptococcus from milk	Indifferent	2	—	+	0.611	1.141	6
14....	Lactic acid bacterium from separator slime	Sour mild	2	+	—	0.637	0.928	6
15....	Fresh cow feces	Sour and cheesy	2	+	—	0.637	1.065	5
16....	" "	" "	3	—	+	0.621	1.065	5
17....	1 c.c. milk after 5 days at 37°	" "	3	—	+	0.655	1.240	8
18....	1 c.c. milk after 2 days at room temp.	" "	2	+	—	1.013	1.169	8
19....	Sewage streptococcus	Sour mild	3	+	—	0.532	1.018	8
20....	Pathogenic streptococcus	" "	5	+	—	0.690	0.995	10
21....	" "	" "	5	+	—	0.611	1.135	12
22....	B. aerogenes 1, University of Chicago	Sour mild	5	+	—	0.646	1.014	13
23....	B. coli A	Sour	5	+	—	0.607	1.310	12
24....	B. aerogenes from milk	Sour and cheesy	4	—	+	0.594	1.096	8
25....	Lactic acid bacterium (Russell)	Sour mild	6	+	—	0.628	0.778	12
26....	" " (Prescott)	" "	7	+	—	0.546	0.546	7
27....	" " (Marshall)	" "	3	+	—	0.568	0.820	8
28....	" " from sour milk	" "	3	+	—	0.542	0.734	7
29....	" 24+28	Sour	2	—	+	0.546	1.057	9
30....	B. aerog. + Lact. acid bact. from milk	"	2	—	+	0.546	0.865	9
31....	B. aerog. (22) + Lactic acid bact. from sour milk	"	2	—	+	0.620	0.987	10
32....	B. coli A + 28	Sour and cheesy	3	+	—	0.681	0.912	6
33....	B. aerog. + sewage streptococcus	Sour	3	—	+	0.599	0.925	11
34....	Milk at room temp.	"	2	+	—	0.921	1.175	5
35....	Milk at 37°	Cheesy	1	—	+	0.995	3.033	8
36....	Milk at room temp.	Sour	2	+	—	0.908	1.196	7
37....	Milk at 37°	Cheesy	1	—	+	0.764	2.846	8
38....	Pathogenic streptoc. at room temp.	Sour mild	2	+	—	0.570	0.691	7
39....	Lactic acid bacteria from separator slime at 37°	Sour mild	2	+	—	0.820	0.873	4
40....	Bacillus from sweet milk at 37°	Sour and cheesy	6	+	—	0.568	0.707	8
41....	" 21+6	Sour and cheesy	2	—	+	0.570	0.672	4
42....	" 5+6	Sour and cheesy	2	+	—	0.556	0.725	9

retained as control, the others were inoculated with various pure cultures and mixtures. Five pathogenic streptococci, a streptococcus from sewage, a streptococcus from cow feces, and eleven lactic bacteria of the Leichmann type (the latter including five varieties obtained from different laboratories), four varieties of *B. aërogenes* from milk and one from the laboratory collection, and a culture of *B. coli* recently isolated from human feces, were inoculated in pure culture in sterile flasks. Several flasks were inoculated with mixtures of pure cultures of each group, two with suspensions of cow manure, and three with sour milk.

A study of the results which appear in the accompanying table, demonstrates that the pathogenic streptococci (2, 3, 20, 21), the streptococci from sewage (19) and cow feces (12), the lactic acid bacteria from Professors Harding (10), Marshall (27), Russell (25), Prescott (26), and the lactic acid bacteria from milk (4, 28), separator slime (5 and 14), and teat wash water (11) produce fermentations in milk, which are identical. The coagulum is homogeneous, no whey appears, and the odor is agreeably, but faintly, sour. The range of time required for coagulation varies, but it has been shown that a sojourn in milk increases the power of rapidly coagulating milk.

Bacilli (6, 7, 8, 22, 23) produce a coagulum, which contracts or breaks up and liberates larger or smaller quantities of whey, accompanied by gas formation. The odor is decidedly sour and cheesy.*

Sterile milk, inoculated with sour milk, ferments like naturally soured milk, and produces the same variation of appearance. Suspensions of cow feces (15 and 16) also produce fermentations, which are indistinguishable from naturally soured milk. Occasionally liquefying streptococci are met with (13), which produce high acidity and at the same time dissolve the casein.

All flasks were titrated for acidity up to the maximum point, i. e., when three successive titrations gave the same result. The highest acidity was reached by naturally soured milk at 37° C. Milk soured at room temperature, and sterilized milk inoculated with mixtures and feces, fermented at approximately the same ratio. Streptococci

* Leichmann has shown that his lactic acid bacterium produces almost pure lactic acid, Hueppe's bacillus (*B. aërogenes*) produces large amounts of volatile acids, hence the difference in odor (*Centralbl. f. Bakt.*, Abth. 2, 1896, 2, p. 777).

of pathological, fecal, and milk origin form a class very similar among themselves in regard to results.

An interesting fact was brought out by comparing the results of the activity of *B. aërogenes* from milk and a mixture of the same organism with a lactic acid bacterium of the streptococcus type. The bacillus alone produced a finely granular precipitate and abundant gas. The mixture produced much less gas, the gas evolution ceasing after a short period of time. The bacillus was crowded out by the non-gas forming streptococcus and thus gas evolution stopped. Under natural conditions both types of lactic acid bacteria may be concerned in the acid fermentation of milk. Leichmann¹ has called attention to this phenomenon by stating that he isolated the Hucppe type from superficial layers where oxygen is readily obtained, the Leichmann type from deep parts of fermenting milk, the latter preferring anaërobic conditions.

That lactic fermentation varies is a matter of everyday experience. Without apparent cause the condition of the coagulum and the amount of whey separated varies in milks of excellent character. This investigation shows that typical lactic acid fermentations can be produced by inoculation of sterilized milk with lactic acid bacteria of either group, or by a mixture of both. The fermentation proceeds in a substantially similar manner as in the natural process, in spite of the chemical and physical alterations in milk which are unavoidable during sterilization. The various appearances of acid fermentations are brought about by various species of bacteria which may bear different numerical relations to each other, and by the variable degree of multiplication according to the temperature at which fermentation takes place. There is, however, a higher degree of acidity produced in naturally soured milk than in sterilized milk inoculated with these two common lactic acid bacteria either in pure culture or in mixtures. This shows clearly that other influences are at work, and in this connection Marshall² has pointed out that other bacteria, especially peptonizing bacteria, exert a favorable influence on the development of lactic acid bacteria.

The lactic acid bacteria of Leichmann, Günther and Thierfelder, Kozai (1), Utz (1), Esten, Schierbeck, and others are described by these writers as follows:

¹ *Loc. cit.*

² *Centralbl. f. Bakt., Abth. 2, 1902, 9, p. 313.*

They are short rods (?) somewhat more than half as broad as long, are oval or frequently with pointed ends, occur in pairs and chains, and are positive to Gram's stain. They are non-motile, do not liquefy gelatin, have neither spores nor capsules. They ferment dextrose and lactose with formation of acid but not of gas. They grow sparingly in ordinary media, much better if dextrose or lactose is added. In stab cultures there is little or no growth on the surface. They grow well in absence of oxygen, some preferring anaërobic conditions. They acidify milk rapidly, form a homogeneous coagulum and decolorize litmus. In non-proteid media no growth is observed. Colonies are small, shapeless and appear preferably in the depth of the jelly.

The characteristics are in perfect accord with those resulting from the studies described. When lactic acid bacteria of the Leichmann type are compared with pathogenic, fecal, or sewage streptococci, it is impossible to detect a difference as far as cultural characteristics have been observed. A careful perusal of v. Lingelsheim's summary of characters of streptococci in Kolle and Wassermann's *Handbuch* demonstrates conclusively that there is no salient difference between recognized streptococci and lactic acid bacteria of the Leichmann type. It may, then, be confidently asserted that there is no organism known which deserves the names *B. acidilactici* or *B. lactis acidilactici*. We can divide the common lactic acid bacteria into two groups. The first group are true bacilli, those described by Pasteur, Hueppe, Clauss, Grotenfelt, Schardinger, Kozai (2), and Utz (2). They are varieties of *B. aërogenes* and may be termed *B. aërogenes var. lacticus*. The second group includes the lactic acid bacteria described by Leichmann, Günther and Thierfelder, Kozai (1), Utz (1), Esten, Schierbeck, and others—all these are called bacilli by the authors—and Grotenfelt's, Weigmann's and Freudenreich's streptococci.¹ Kruse² has suggested the relation of the latter group to streptococci although no experimental evidence is reported, and proposed the appellation *Streptococcus lacticus*.

A general summary of the results leads to the following conclusions:

1. *Bacillus acidilactici* is a myth. The ordinary bacteria producing lactic acid fermentation in milk are *B. aërogenes var. lacticus*, and *Streptococcus lacticus*. The possibility of *B. coli* and other forms participating in milk fermentation is not excluded.

2. *Streptococcus lacticus* agrees in morphological, cultural, and coagulative properties with pathogenic, fecal, and sewage streptococci.

¹See MIGULA, *System der Bakterien*, 1900, 2, pp. 18, 39, 42.

²*Centralbl. f. Bakt., Abth. 1*, 1903, 34, p. 737.

3. *Streptococcus lacticus* can be detected in cow feces, on the external surfaces of cows, and in milk at all stages of subsequent handling.

4. Souring of milk is caused by co-operation of both groups of lactic acid bacteria and may be aided by some peptonizing bacteria always present in market milk.

5. Gas is produced by *B. aërogenes* var. *lacticus*, but as a rule this organism is held in check and ultimately stopped by the presence and final ascendancy of *Streptococcus lacticus*.

6. Acid is produced during lactic acid fermentation of milk by both classes of organisms to a marked degree. *B. aërogenes* var. *lacticus* is more sensitive to the presence of acid than *Streptococcus lacticus*. This results in the presence of *B. aërogenes* in large numbers in initial stages of fermentation, *Streptococcus lacticus* becoming master of the field in terminal stages.

7. Artificial lactic acid fermentation in sterilized milk can be produced by inoculation of pure cultures of bacteria of either group, or better by the two groups combined.

8. Since *Streptococcus lacticus* is invariably present in market milk and in fresh milk collected with good precautions, the sanitary significance of streptococci in market milk will need further investigation.

In conclusion I wish to thank Professor Edwin O. Jordan, under whose direction this work was carried on, and Dr. Norman Mac L. Harris for valuable advice and encouragement during the progress of the work.

DESCRIPTION OF PLATES.

PLATE 3.

Streptococcus pyogenes from tonsil in scarlet fever.

FIG. 1.—From serum broth.

FIG. 2.—From lactose broth.

FIG. 3.—From litmus milk.

PLATE 4.

Streptococcus lacticus from the Massachusetts Institute of Technology.

FIG. 1.—From serum broth.

FIG. 2.—From lactose broth.

FIG. 3.—From litmus milk.

PLATE 5.

Streptococcus lacticus from Chicago milk.

FIG. 1.—From serum broth.

FIG. 2.—From lactose broth.

FIG. 3.—From litmus milk.

PLATE 3.

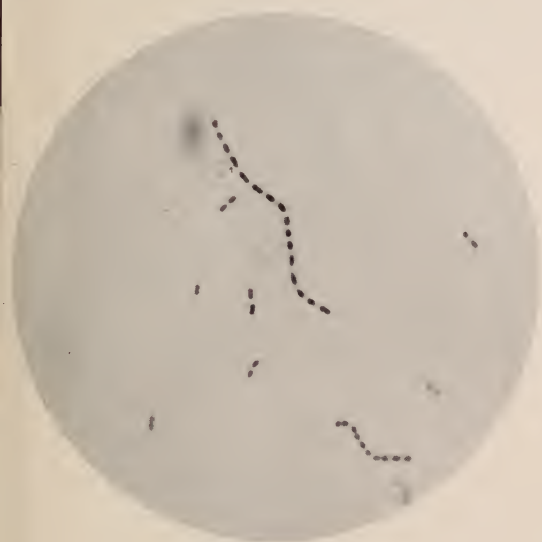


FIG. 1.

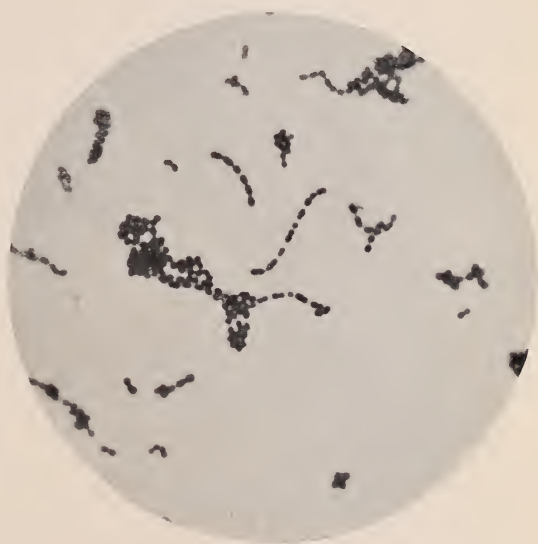


FIG. 2.



FIG. 3.

PLATE 4.

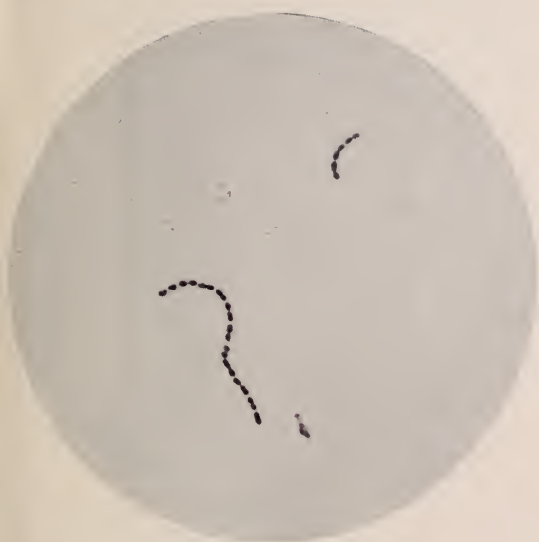


FIG. 1.



FIG. 2.



FIG. 3.

PLATE 3.

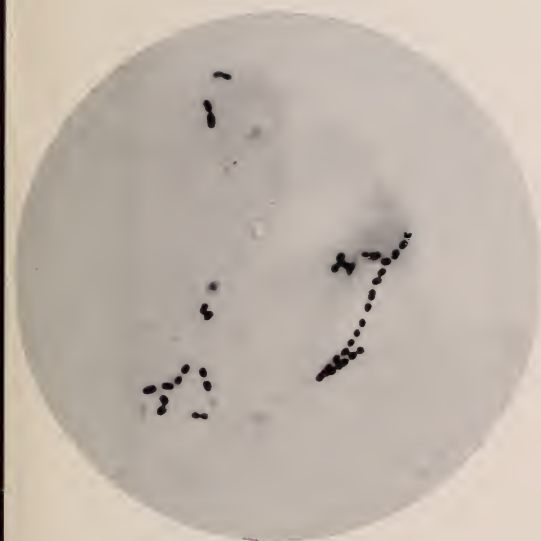


FIG. 1.



FIG. 2.



FIG. 3.

A METHOD OF ISOLATING THE PNEUMOCOCCUS IN MIXED CULTURES, SUCH AS THROAT CULTURES.*

GUSTAV F. RUEDIGER.

(From the Memorial Institute for Infectious Diseases, Chicago.)

ABOUT a year ago Hiss¹ published a paper in which he recommended inulin as an aid in differentiating the pneumococcus from the streptococcus. He showed that pneumococci ferment inulin, when added to a sugar-free culture medium which is favorable for the development of these organisms, while streptococci fail to ferment it. This work has since been taken up by several investigators in this country, and it has been found that Hiss's observations are correct, with perhaps one exception. There is a group of organisms² which appear to be identical with the pneumococcus, both morphologically and culturally, but they do not ferment inulin. In spite of this fact, however, it must be stated that the inulin reaction is often a convenient method of determining to what class a certain organism belongs.

A number of methods are in use for isolating pneumococci from the mouth in health and in disease. One of these is by animal inoculation. Usually a rabbit or a white mouse is inoculated with a quantity of sputum, and if the animal dies the blood and internal organs are examined for encapsulated diplococci. If the animal does not die, the experiment does not show much, because pneumococci may have been present, but in too small a number, or their virulence may have been too low to produce death. Various plate methods are also in use, but of these I shall mention only the blood-agar plate method. It is well known that in this medium *Streptococcus pyogenes* forms small gray colonies, which are surrounded by a zone of hemolysis, while the pneumococcus and *Streptococcus viridans* of Schottmüller³ form green colonies. To isolate pneumococci from blood-agar plates which have been inoculated with material from throats, it is necessary to make subcultures from at least 10 to 15

*Received for publication January 25, 1906.

¹*Jour. Exper. Med.*, 1905, 7, p. 317.

²See PARK AND WILLIAMS, *Jour. Exper. Med.*, 1905, 7, p. 403; also DUVAL AND LEWIS, *ibid.*, p. 473.

³*Munch. med. Wchnschr.*, 1903, 50, pp. 849 and 909.

green colonies. In this way I hardly ever failed to get at least one culture that ferments inulin, and from some throats I have obtained as high as eight inulin fermenters out of ten cultures tested. Although this method gives fairly satisfactory results, it involves a large amount of useless labor. With the object of making the isolation of pneumococci easier, I have prepared a blue litmus inulin-agar medium, in which pneumococci form red colonies. This medium is simply a sugar-free agar, with the addition of inulin and litmus, and is made as follows:

(a) Peptone (Witte)	10
Agar-agar	15
Sugar-free beef broth (neutral)	1,000

Dissolve by boiling one hour, adding water from time to time to make up the loss from evaporation. Heat in the autoclav for 15-20 minutes (to prevent subsequent precipitation while sterilizing), clarify with egg, filter through cotton, and make the volume up to 800 c.c. with distilled water.

(b) Dissolve 15 grams of pure inulin in 200 c.c. of distilled water, mix this solution with (a), add 20 c.c. of a 5 per cent solution of litmus (Merck's highest purity), tube, and sterilize in the autoclav under 10 pounds pressure for 15 minutes. Each tube should contain 7 or 8 c.c. of medium. Some pneumococci do not grow well in this medium, hence it is necessary to add 1 c.c. of heated (65° C.) ascites fluid to each tube of melted agar (which has been cooled to 45°) immediately before using.

In this mixture the pneumococci grow very well, and in 24 to 96 hours the inulin fermenters form red colonies (acid production), which stand out very prominently on the blue background. The surface colonies do not produce as much acid as the deep colonies, and it is for this reason that each tube should contain a rather large amount of medium. In this way a thick plate is formed, with relatively few surface colonies.

The pneumococci are practically the only mouth bacteria that ferment inulin. I have tested four strains of *Staphylococcus aureus*, 20 strains of *B. pseudodiphtheriticus*, 120 strains of *Streptococcus pyogenes*, one strain of *Micrococcus catarrhalis*, one strain of *B. mucosus*, and one strain of *Micrococcus tetragenus*; not one of these fermented inulin. Among 10 strains of diphtheria bacilli, I encountered one which ferments inulin. I have studied in detail 22 cultures, made from red colonies in plates of this medium which had been inoculated with material from the throats of five pneumonia patients, six scarlet-fever patients, and one case of pharyngitis. All of these

organisms are Gram-positive, lanceolate, oval, or rounded cocci, which grow chiefly in pairs on blood agar and serum agar, but are also found in short chains in liquid media. All of them ferment inulin,* and all but four form green colonies in blood-agar plates. Capsules could be demonstrated on 13 cultures, while two were doubtful. The details are shown in the accompanying table:

TABLE 1.

SHOWING THE CHARACTERISTICS OF ORGANISMS ISOLATED FROM THROATS BY MEANS OF LITMUS INULIN-AGAR PLATES.

Organism	Source	MORPHOLOGY		Colonies in Blood-Agar Plates	Capsules
		On Blood-Agar Slants	In Calcium Broth		
1	Pneumonia Throats	Round and oval cocci in pairs and chains	Diplococci		+
1a		Lanceolate diplococci	Diplococci	Green	-
2		Lanceolate diplococci and short chains.	Diplococci and chains	Slightly hemolyzing. Not green	+
2a		Lanceolate diplococcus and short chains	Diplococci	Small, brownish	-
3		Lanceolate diplococci	Short chains and diplococci	Green	+
3a		Diplococci	Diplococci	Small, brownish	-
4		Lanceolate and round cocci in pairs	Diplococci and short chains	Green	+
4a		Lanceolate and round cocci in pairs	Diplococci and short chains	Green	-
5		Round diplococci	Short chains and diplococci	Green	?
5a		Chiefly short chains. Some diplococci	Short chains and clumps	Slightly hemolyzing	-
6	Scarlet-Fever Throats.	Diplococci	Diplococci	Green	+
7		Lanceolate diplococci	Diplococci	Green	+
7a		Lanceolate diplococci	Diplococci	Green	+
7b		Lanceolate diplococci	Diplococci	Green	+
8		Lanceolate and round cocci in pairs	Short chains and diplococci	Green	+
9		Lanceolate diplococci and short chains.	Diplococci	Green	+
9a		Lanceolate diplococci	Diplococci	Green	+
10		Lanceolate diplococci	Diplococci	Green	-
10a		Lanceolate diplococci	Diplococci and short chains	Green	+
11		Lanceolate diplococci	Diplococci	Green	-
11a		Lanceolate diplococci	Chains	Green	?
12	Pharyngitis	Lanceolate diplococci	Chains and diplococci	Green	-

Some pneumococci isolated by this method are somewhat atypical in that they tend to form chains and have no capsules. Fourteen

*The inulin-fermenting power was tested in the following medium; (a) Take 300 c.c. of rich, sterile ascites fluid, add 100 c.c. of sterile distilled water, and heat to 65° to 70° for one-half hour. (b) Dissolve 6 grams of inulin and 6 grams of peptone (Witte) in 200 c.c. of distilled water, add 6 c.c. of a 5 per cent litmus solution, and sterilize in the autoclave under 10 pounds pressure for 15 minutes. Mix (a) with (b), tube under aseptic precautions, and incubate the tubes 24 hours before using. This medium is very favorable for the growth of pneumococci, but care must be taken not to use ascites fluid that contains fermentable carbohydrates.

rabbits were inoculated with large doses of these organisms but only five of the animals died. In the animal body these organisms grew almost exclusively in pairs and three of the five strains produced capsules.

In routine examination of throats for pneumococci the following scheme has been found very satisfactory. A sterile cotton swab is rubbed against the tonsils and walls of the pharynx. The swab is rinsed in 1 c.c. of sterile broth, and four or five tubes of litmus inulin-agar are inoculated with this broth and plated. The plates are incubated, and examined for red colonies in 24 hours. If no red colonies are found, the plates must be examined daily for four days, as some pneumococci ferment inulin rather slowly, and red colonies may appear as late as the fourth day.

NOTES ON THE COMMON MOSQUITOES OF BIHE AND
BAILUNDO DISTRICTS, PORTUGUESE WEST
AFRICA.*

FREDK. CREIGHTON WELLMAN.

(Concluded from Vol. 2, p. 631.)

Culex hirsutipalpis, Theob.

Mono. Culicid., 1901, 1, p. 378. *Gnats or Mosquitoes*, 2d ed., 1902, p. 403.
Entomologist, 1905, 38, p. 156.

A LARGE mosquito which has as its striking feature the long, dense, black hairs on the last two joints of the ♂ palpi. It closely resembles *C. plumosis*. Thorax golden brown. Two cream-colored spots on the mesonotum. There is a darkish line down the middle of the thorax. Abdomen dark brown with semi-circular, light-yellow, basal, median, and lateral spots. Proboscis with broad yellow band. Legs brown with cream-colored bands on the tibio-metatarsal and tarsal joints. The wings have the first submarginal cell somewhat longer and narrower than the second posterior. A peculiarity of the wings is the very distinct incrassations (the so-called sixth and eighth veins). The figure of

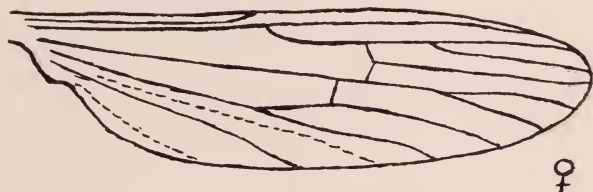


FIG. 6.—Wing Venation of *C. hirsutipalpis* ♀.

the ♂ unguis in Theobald's monograph is incorrect. He has been able to clear-up this point by dissection of material sent by the writer. The ♂♂ from this region differ from the type in having no pale band at the apex of the palpi, and Mr. Theobald's diagnosis of my first specimen was "*Culex* sp. nov. near *hirsutipalpis*, Theob." The eggs are black and laid in large rafts. A rainy-season mosquito. Breeds in enormous numbers in dirty pools resulting from building, grading, etc. Breeds out readily in captivity. I have kept them for two generations. They vary greatly in size. The type measures 5 mm., but some individuals are a third smaller than this.

*Received for publication February 5, 1906.

GENERAL REMARKS ON THE GENUS *HEPTAPHLEBOMYIA*, THEOB. AS
ILLUSTRATED BY THE WRITER'S SPECIMENS OF *H. SIMPLEX*.

Heptaphlebomyia simplex, Theob.

Mono. Culicid., 1901, 3, p. 337. *Entomologist*, 1905, 38, p. 157.

In view of Mr. Theobald's remark that the fresh "material sent . . . by Dr. Creighton Wellman has enabled me to add fresh generic characters to those already given," it may be of interest to recall here the main points in his new characterization of the genus. Head clothed with narrow curved scales and upright forked ones except at the sides, where they are small and spatulate. Palpi of ♀ small but prominent, in ♂ acuminate, the last two segments hairy. Thorax, scutellum, and prothorax clothed with narrow, curved scales. In ♀♀ the pleuræ have pointed patches of flat scales, which in the ♂♂ are apically rounded. Wings with typical *Culex* venation, except the seventh long, scaled vein in ♀. In the ♂ there is no true scaled seventh vein, but the sixth is bent at right angles near the edge of the wing. The scaled seventh vein in the ♀ and the characteristic scales on the pleuræ are unique among *Culicidae*.

The species *simplex* (which so far is the only one in the genus) has hitherto been known only from ♀ specimens. The ♂ has now for



FIG. 7.—Wing Venation of *H. simplex* ♀.

the first time been described from three individuals collected by the writer. *H. simplex* can, as has been said, easily be told from all other mosquitoes by the ♀ having a distinct, scaled seventh wing

vein. It is a remarkable fact that the ♂ does not share this peculiarity. The general facies of *H. simplex* suggests *C. fatigans* while the leg-markings simulate those of *C. creticus*. The thorax and pleuræ are brown, the latter with snowy white dots. The abdomen is dark brown with basal, light, curved bands. There are lateral segmental spots in the ♀, while the ♂ has scattered yellow scales on the apical segment and pale bands on the base and sides of the penultimate segment. The wings in both sexes have the first submarginal cell longer

¹ *Entomologist*, loc. cit., p. 156.

and narrower than the second posterior. Like the preceding it is a rainy-season mosquito. The eggs are brownish and are laid in rafts. Will breed in foul pools. Length 3.5-4 mm.

SUPPLEMENTARY NOTE ON *MANSONIA AFRICANA*, THEOB. AND *CULEX VIRIDIS*, THEOB.

Cf. *Mono. Culicid.*, 1901, 3, pp. 212 and 273 f.

The mosquito which was determined as *M. africana* (*vide supra*) is not a very common mosquito in the district where I first took it (Bihé), but I have since found it in enormous numbers in a district about a hundred miles from the first named (S. Bailundo), and have had opportunity to compare a suite of specimens with the descriptions of Theobald's *M. uniformis*; and I think with him that they are the same and that the name *M. africana* should sink. Also on re-reading my remark on *C. viridis* I note that my language would convey the impression that the imago is green. The larva and pupa are referred to. The imago itself is brown except the pleuræ, which are greenish gray. I was breeding out larvæ which had been brought me from different sources and all put into one vessel by the collector when I wrote the note mentioned, and the bright green larvæ and pupæ of *C. viridis* were very striking. There is another green larva which sometimes occurs with those of this mosquito. On breeding these out I find they are not *C. pulcher*, as I surmised above, but are a species unfamiliar to me, which I am forwarding for determination. In the course of larva-hunting, since writing the original note on *C. viridis* I have been able to verify my suspicions then stated and find that it breeds in certain *Bromeliaceae*, notably several species of *Amomum* and *Costus*.

It should be borne in mind that the *Culicidae* mentioned in the preceding list are confined to those which are common over the two somewhat extensive districts studied—Bihé and Bailundo. Other mosquitoes, abundant in limited areas, together with a few rare species, will be discussed in a subsequent paper. A full consideration of the pathological significance of these gnats would be of interest but is out of place in a study of this kind. I may say, however, that of the anophelines the most important is *M. junesta*, although I have proven *P. austeni* to be also also a carrier of malaria. I am elsewhere pub-

lishing statistics in the form of charts and maps which show the geographical distribution of the former mosquito in this region to be essentially the same as that of severe malaria and black-water fever, and that the amount of rainfall at different seasons of the year has a direct relation, both to the comparative number of anophelines taken and the percentage of malarial infections among the natives.

THE TOXINS AND ANTITOXINS OF POISONOUS MUSHROOMS (*AMANITA PHALLOIDES*).*

WILLIAM W. FORD.

(From the Bacteriological Laboratory, Johns Hopkins University.)

CONTENTS.

1. HISTORICAL INTRODUCTION.
 - Pathological Changes.
 - Clinical Symptoms.
 - Chemical Investigations.
2. PREPARATION OF MATERIAL.
3. THE HEMOLYSIN PRESENT IN *Amanita phalloides*.
 - Properties of the Hemolysin.
 - Inactivation.
 - Attempted Reactivation.
 - Reactions with
 - a) Resistant Corpuscles.
 - b) Heated Sera.
 - c) Endocomplement.
 - d) Lecithin.
 - Combinations of Resistant Corpuscles with *Amanita phalloides*.
 - Reactions with Lymphocytes.
 - Natural Antihemolysins.
 - Reactions with Cholesterin.
 - Summary of Hemolytic Reactions.
4. ACTION OF *Amanita phalloides* UPON ANIMALS.
 - a) Postmortem Changes.
 - b) Microscopic Examination.
 - c) Protocol of Animal Inoculation and Autopsy.
 - Estimation of Minimum Fatal Doses.
5. IMMUNIZATION OF ANIMALS.
 - Antihemolytic Properties of Immune Sera.
 - Antitoxic Properties of Immune Sera.
 - Cellular Immunity.
6. CONCLUSIONS.
7. BIBLIOGRAPHY.

I. HISTORICAL INTRODUCTION.

The deaths annually from consumption of poisonous fungi, generally known as toadstools, are so numerous, especially in countries where fungi are an important and valuable constituent of the food of the poorer classes, while therapeutic measures are so notoriously use-

*Received for publication February 28, 1906.

less in the treatment of the severe cases, that investigations into their toxicological properties always have practical importance. The actual mortality from this cause is difficult to estimate. There are, however, a number of notable individuals in early times who lost their lives from eating poisonous mushrooms, among whom may be mentioned the daughter of the historian Pausanias, the emperor Claudius, King Charles VI of France, the pope Clement VII, and the Princess of Conti, while in modern times the most prominent example is the Czar Alexis of Russia. For over a century fatal cases have been reported in medical periodicals, and during the latter part of the 18th century Paulet¹ found 100 deaths from this cause in the vicinity of Paris. Later Bardy² collected 60 cases in the Vosges, covering a period of six years. In 1885 Guillaud³ estimated the annual number of deaths in the southwest of France at about 100 cases.

In Japan fatalities from fungus poisoning are numerous, Inoko⁴ having reported 481 cases in a period of eight years. In other countries they are frequent, for Falck⁵ has collected 53 in Germany with 40 deaths, and there are numerous reports of individual groups in the German literature.

In America, Palmer,⁶ of Boston, has found 33 cases with 21 deaths, and Forster,⁷ of Charlestown, 44 cases with 14 fatalities.

Such statistics do not represent adequately the real loss of life, since the majority of cases do not find their way into medical literature. Thus during the past summer there were two cases in Baltimore, fortunately neither fatal; a family was poisoned in Cleveland, Ohio, with two deaths; nine persons in Fostoria, Ohio, with several deaths; ten individuals in Toronto, with two deaths; and Pfromm⁸ of Philadelphia has just reported the poisoning of four members of an Italian family with four deaths. In addition Plowright⁹ has recently reviewed one of his earlier cases to which he has added three other deaths in England.

The three most important varieties of poisonous fungi are *Amanita muscaria*, *Helvella esculenta*, and *Amanita phalloides*. From the first of these, *Amanita muscaria*, known as the "Fly Amanita," from its extensive use as a fly poison, Schmiedeberg¹⁰ and his pupils, Koppe and Harnack,¹¹ have extracted the alkaloid muscarin, which they have shown by extensive animal experimentation to be respon-

sible for the symptoms and lesions found in cases of muscarin poisoning in man. Later muscarin was prepared synthetically by the oxidation of cholin, and its chemical constitution has been estimated as $C_5H_{15}AZO_3$. Artificial muscarin differs materially from the natural product, in its action upon animals. The *Amanita muscaria* is employed in large quantities by the peasants of the Caucasus, who prepare an intoxicating beverage, from the excessive use of which many individuals die annually. Poisoning from eating *Amanita muscaria* is comparatively rare, since this fungus has a bitter, unpleasant taste, and in consequence is not eaten in any great quantity. The cases seldom terminate fatally, and the symptoms are referable to the profound action of muscarin upon the nerve centers. It has been shown experimentally that atropin is a perfect physiological antidote for muscarin, and the use of this drug has frequently been found of great value in severe cases of poisoning. (See the report of Prentiss¹² concerning the poisoning of the Count de Vecchi and his physician, Dr. K., the latter of whom recovered from muscarin poisoning after treatment with excessive doses of atropin.)

The second poisonous fungus, *Helvella esculenta*, owes its toxicity to helvellic acid, a substance isolated by Boehm and Külz,¹³ who give it the structural formula $C_{12}H_{20}O_7$, and who have shown that it is strongly hemolytic in its properties. Numerous fatalities among the German peasants were formerly attributed to this fungus, but very few deaths have been reported within recent years for which *Helvella esculenta* could be held definitely responsible.

The most important of all the poisonous fungi, because of its great abundance, its superficial resemblance to the edible mushroom, its innocent appearance, its delicious taste, and, finally, its extreme toxicity is the *Amanita phalloides*, the small or spring form of which is called the *Amanita verna*.

This is a pure white fungus growing in damp woods, and occasionally appearing in open sunny fields which abound in meadow mushrooms, with which it is rarely mixed. Its botanical characters are well marked. It varies in height from four to six inches, with a top or pileus about two and one-half inches in diameter. The gills and the attached spores are white. The stalk is white and slightly

fibrous. About the upper part of the stalk is a definite veil, and the base of the stalk is surrounded by a large cup or volva sunk deep beneath the surface of the ground. This cup is known as the "death cup," and its bulbous shape has given the fungus its French name, *Amanita bulbeux*.

The pileus is usually pure white, but forms have been described where the pileus is colored green, yellow, or brown. The white forms are by far the most common, at least in this country.

The small form, the *Amanita verna*, grows to a height of two or three inches, with a pileus not more than one inch in diameter. The toxic properties of the *Amanita verna* have been so well recognized in France that Bulliard,¹⁴ the most eminent of French mycologists, nearly a hundred years ago gave it the name "destroying angel." In England and in America the *Amanita phalloides* is known as the "Deadly Amanita."

The deadly amanita is of extreme toxicity. Plowright¹⁵ has reported the death of a child of 12 years from eating a third of the top of a small plant, while there are many cases on record where adults have perished from eating two or three good-sized specimens. Indeed, a long study of the literature of fungus poisoning leaves little room for doubt that practically all the deaths from mushrooms are to be attributed to this one species.

The deaths in France from the consumption of *Amanita phalloides*, were collected in 1900 by Gillot,¹⁶ who reports 123 fatalities in which the fungus eaten was definitely identified. In addition to Gillot's cases I have been able to find in the German, Italian, and English literature, and in the French since 1900, nearly 200 deaths from the same fungus, so that there are a sufficient number of cases reported in detail to give us accurate knowledge of their symptoms during life and of their postmortem lesions.

PATHOLOGICAL CHANGES.

The earliest careful study of the pathological changes found in phalloides poisoning was made by Maschka,¹⁷ who summarized these changes as:

1. Lack of postmortem rigidity;
2. Widening of the pupils;

3. Failure of the blood to coagulate, and a cherry-red color;
4. Ecchymoses and hemorrhages in the serous membranes and parenchymatous organs;
5. Dilatation of the bladder with urine.

Later Studer Sahli and Schärer,¹⁸ of Bern, contributed a valuable series of papers to the subject of phalloides poisoning, treating it from the botanical, clinical, and pathological standpoints. They confirmed all of Maschka's observations, calling attention, to the extensive necrotic and fatty changes in liver, kidney, heart, and voluntary muscles. These lesions Maschka had seen in three of his seven cases, but had not considered essential.

The fatty degeneration of the organs and tissues is pronounced, and the amount of fat in the liver is extremely great, almost as abundant as in phosphorous poisoning. Thus Tappeiner¹⁹ has estimated the fat content of the liver in two cases of *Amanita phalloides* poisoning as 53.6 per cent and 68.9 per cent as compared with a fat content in cases of phosphorous poisoning of from 50 to 70 per cent. The widest variation in the fat of normal livers is from 8.9 to 25.5 per cent (Perls). The most valuable confirmation of the work of Maschka, Studer Sahli and Schärer and Tappeiner has been that of Moers,²⁰ who examined the internal organs of three individuals dead of *Amanita phalloides* poisoning, testing them for arsenic, phosphorus, and muscarin. All of these substances were absent, so that any doubt as to the relationship between the fungus and the post-mortem lesions was dispelled. In Moers's cases the microscopic changes present were identical with those described by the earlier observers.

CLINICAL SYMPTOMS.

The clinical symptoms following the ingestion of *Amanita phalloides* are characteristic. After a latent period of from six to twelve hours, during which the victims remain quite well, they are suddenly seized with terrible abdominal pain, with excessive vomiting and thirst. Diarrhea may set in with mucous, bloody stools, or the bowels may be constipated. Anuria is usually present. The paroxysms of pain may be so severe as to result in a peculiar Hippocratic facies, emphasized by the French as "la face vultueuse."

Periodical attacks of pain and vomiting alternate with periods when the symptoms seem ameliorated. The patients rapidly lose strength and frequently become jaundiced. After three to four days in children, and six to eight days in adults, coma develops, from which the patients cannot be aroused by any artificial stimulus, and which results in death within a few hours. Cyanosis and lowered temperature precede the fatal exit. Ocular symptoms and convulsions do not ordinarily occur, but convulsions may be present as a terminal event. The mortality in various epidemics is high, varying from 60 to 100 per cent, and depending largely upon the amount of the fungus eaten. Treatment is useless.

CHEMICAL INVESTIGATIONS.

The earlier chemical investigations upon *Amanita phalloides* were conducted by Boudier,²¹ who isolated a substance in 1866 which he considered an alkaloid and which he named "bulbosin." A year later Latellier and Speneux²² obtained a preparation which they named "amanitin," and which they believed to consist of an irritating and a narcotic principle. Finally Oré²³ extracted a substance called "phalloidin," which he later endeavored to prove was identical with strychnin.

It is impossible to come to any definite conclusion concerning bulbosin, amanitin, and phalloidin. None of these substances was obtained in a pure condition; the fungi employed for the investigations were mixtures of a number of different varieties, including *Amanita muscaria*, and it is generally believed by mycologists that the products mentioned were impure preparations of muscarin or cholin.

At all events, Kobert's²⁴ investigations in 1891 cleared up a great deal of the obscurity surrounding the matter. He collected a large quantity of fungi which were accurately identified as *Amanita phalloides*. From these fungi he obtained a substance characterized by its marked hemolytic action, his dried extract dissolving ox blood in a dilution of 1:125,000. To this hemolytic principle, extracted from *Amanita phalloides*, Kobert gave the name "phallin," and from its thermolability he considered it a toxalbumen. By intravenous injection with phallin Kobert produced intracorporeal laking

of the blood, and attributed its toxic action to its hemolytic properties alone.

Since Kobert's investigations little of value has appeared in the literature.

Seibert,²⁵ working in Kunkel's laboratory, was unable to confirm Kobert's conclusions, but the value of Seibert's work is destroyed by the fact that he used a yellow fungus, *Amanita citrina*, which is seldom, if ever, toxic to man. His methods of extracting the fungus were not such as to bring out any hemolytic properties and he made no microscopic examinations of his experimental animals, so that we do not know whether the changes produced by other hemolytic agents, such as pigmentation of the spleen, were present or not.

As far as can be learned from a review of the literature no serious attempt has been made to study *Amanita phalloides* from the standpoint of serum-therapy, so in view of the great theoretical and practical interest which attaches to the subject of toxins and anti-toxins at the present time, it was determined to carry out a systematic investigation of poisonous properties with the object of determining whether antitoxins could be prepared for it. These investigations have now been pursued three years and it is the object of the present paper to report the most important of the results.

2. PREPARATION OF MATERIAL.

The fungi were collected in considerable quantity in the Blue Ridge Mountains of North Carolina, of Maryland, and of Pennsylvania, as well as in some of the thickly wooded regions in the vicinity of Baltimore. Only those specimens which corresponded to the standard botanical description of *Amanita phalloides* given by Atkinson,²⁶ Farlow,²⁷ Peck,²⁸ and others, were saved, and of these only the pure white forms have thus far been employed. The color of the pileus and its freedom from scales, the color of the spores, the fibrous character of the stem, the presence of a veil, and finally the presence of a poison cup at the base of the stem were considered essential characters in identification.

The fungi were dried either in direct sunlight or in especially prepared ovens, and the dried material was then preserved in the dark. A number of unsuccessful attempts were made to extract the

fungi satisfactorily, during which much of the first year's collection was wasted. Finally a satisfactory method was elaborated and the same method has been followed throughout the investigations. The dried fungus is first carefully weighed and pulverized in a mortar. A definite quantity of water is now added and the material placed *on ice for 48 hours*. It is then expressed under considerable pressure, the juice thus obtained being dark brown in color with an odor similar to that of the fresh plant. At least three extractions are made from each lot of fungi. The extract is now filtered, first through fine filter paper, then through a Berkefeld filter. For hemolytic reactions concentrated NaCl solution is added in quantities sufficient to bring the salt content of the extract up to 1 per cent. The extract may be preserved almost indefinitely, but a small piece of thymol is usually added to each flask to prevent the development of molds.

As thus prepared, the extracts differ slightly from those made by Kobert. It being highly desirable to obtain all the constituents of the fungus and to preserve them intact, the extract was not evaporated to dryness lest some injury be done to any delicate poisons present. Evaporation, moreover, leaves a viscid, sticky mass which absorbs water so rapidly that it is impossible to measure exact quantities; so both for hemolytic work and for the inoculation of animals, the fluid extracts are preferable.

3. THE HEMOLYSIN PRESENT IN *AMANITA PHALLOIDES*.

The extract of *Amanita phalloides* is a powerful hemolytic agent. It quickly dissolves the erythrocytes of guinea-pig, rabbit, fowl, pigeon, dog, goat, and man. This reaction takes place rapidly at 37° C., somewhat more slowly at lower temperatures. The corpuscles are not agglutinated, and both washed and unwashed corpuscles are alike dissolved. The reaction takes place with considerable dilution of the crude extract as can be seen from Table 1.

The same solution of *Amanita phalloides* exerts practically the same action upon washed guinea-pig corpuscles. In the following table the corpuscles were washed several times in 0.75 per cent NaCl solution. The hemolysis is considerably more active with the

washed corpuscles than with the unwashed, but the difference cannot be expressed in figures.

TABLE 1.
LYSIS OF GUINEA-PIG BLOOD (UNWASHED).

Amanita phalloides Extract I, prepared from 8 grams dried *Amanita* and 100 c.c. distilled water. Soaked 48 hours on ice, expressed under pressure, filtered through filter paper and then through Berkefeld filter. Concentrated NaCl solution added to make the solution 1 per cent NaCl.

Extract I + 5 per cent Blood 1.0 c.c.		Lysis
Extract 1.0 c.c.	Complete
" 0.1	"
" 0.006	"
" 0.004	Almost complete
" 0.002	Partial
" 0.001	None
NaCl Sol. only (control)	"

Observations made at the end of one, two, and five hours. Final readings taken at the end of 18 hours. The same procedure followed in all hemolytic experiments.

The corpuscles are not agglutinated.

TABLE 2.
LYSIS OF GUINEA-PIG BLOOD (WASHED)

Extract I + 5 per cent Blood 1.0 c.c.		Lysis
Extract 1.0 c.c.	Complete
" 0.1	"
" 0.01	"
" 0.006	"
" 0.004	Almost complete
" 0.002	Partial
" 0.001	None
NaCl Sol. only (control)	"

From these two tables it may be seen that complete solution of both washed and unwashed guinea-pig corpuscles occurs with this extract of *Amanita phalloides* in quantities representing 0.006 c.c. The same extract tested upon rabbit corpuscles is somewhat less powerful, dissolving these erythrocytes completely in a dilution of 1 to 100 or 0.01 c.c. The washed and the unwashed corpuscles are dissolved with practically the same quantities of the extract. Erythrocytes from various species of animals exhibit great differences in susceptibility to *Amanita phalloides* as they do to most hemolytic agents. Thus Extract I which dissolves guinea-pig blood in a dilution of 0.006 c.c. and rabbit blood in a dilution of 0.01 c.c. has much less action upon fowl, dog, and pigeon corpuscles which are completely dissolved by 0.06 c.c., 0.08 c.c., and 0.2 c.c., respectively. With dog blood an abundant, brown, flocculent precipitate is given by

Amanita phalloides extracts, and the appearance of this precipitate marks the limits of hemolysis. Dog serum does not give this reaction, but washed corpuscles, freed from serum and then laked with minimum quantities of water give it abundantly. It is apparently some reaction between the phalloides extract and the albuminous constituents of the dog erythrocytes, while at the same time some reducing action may be exerted upon the oxyhemoglobin. The same flocculent precipitate appears with pigeon blood, and here, too, this marks the limits of hemolysis. This precipitate also is given by laked pigeon corpuscles. When examined microscopically the nuclei of the pigeon corpuscles are always found intact.

The following table gives a synopsis of the hemolytic reactions of Extract I, and the relative susceptibility of various erythrocytes.

TABLE 3.
SYNOPSIS EXTRACT I.

Species	Quantity of Extract I Necessary to Cause Complete Solution of 1 c.c. of 5 per cent Blood Suspension
Guinea-pig	0.006 c.c.
Rabbit	0.01
Fowl	0.06
Dog	0.08
Pigeon	0.2

Other extracts of *Amanita phalloides* have been obtained which are considerably stronger than the one mentioned above. Thus an extract was obtained from 15 grams powdered *Amanita phalloides*, and 200 c.c. distilled water, which dissolved guinea-pig corpuscles in a dilution of 1:1000 and rabbit corpuscles in a dilution of 1:500. This extract was tested carefully upon human blood corpuscles which were found relatively susceptible, as can be seen in the following table, which gives the result obtained with washed and unwashed corpuscles:

TABLE 4.
LYSIS OF HUMAN BLOOD.

Extract IV +5 per cent Blood 1.0 c.c.	Lysis Complete
Extract 1.0 c.c.	"
" 0.1	"
" 0.05	"
" 0.03	"
" 0.02	"
" 0.01	None
NaCl only (control)	"

Hemolytic strength for human blood 0.02 c.c.

This extract was also studied with a variety of other erythrocytes, including goat, sheep, swine, and beef. Goat blood was found quite resistant, 0.5 c.c. of the crude extract being necessary to dissolve completely 1 c.c. of a 5 per cent suspension. Swine, sheep, and beef blood were found insusceptible to this particular preparation.

The following table gives a synopsis of the reactions of this extract.

TABLE 5.
SYNOPSIS EXTRACT IV.
Quantity of Extract IV Necessary
to Dissolve Completely 1 c.c. of
5 per cent Suspension of Blood

Species					
Guinea-pig	0.001 c.c.
Rabbit	0.002
Man	0.02
Goat	0.5
Swine	Negative
Beef	"
Sheep	"
					Some discoloration of corpuscles
					"
					"
					"

PROPERTIES OF THE HEMOLYSIN.

From these experiments it can be seen that the hemolytic ingredient of *Amanita phalloides*, corresponding to the phallin of Kobert acts upon a variety of erythrocytes in a characteristic manner. Certain corpuscles, like those of guinea-pigs and rabbits, are very susceptible to its action while others are relatively resistant. Important questions at once arise as to the nature of this hemolytic material. Does it belong to the class of hemolytic principles found in normal blood serum, or in the blood serum of animals treated with alien corpuscles, which have been shown by Bordet and Ehrlich and their followers to consist of two substances, one uniting with the corpuscles (amboceptor, immune body, substance sensibilatrice), and another bringing about their solution (complement, alexin)? Or does it correspond to the bacterial hemolysins whose action is apparently exerted directly upon the susceptible erythrocytes without the intermediation of any complemental substances? Finally, can one be dealing with a hemolytic principle similar to that found in cobra venom, which was shown by Flexner and Noguchi²⁹ to belong to the class of serum hemolysins in that its hemolytic activity is dependent upon the intra-action of two substances, amboceptor and complement, but in which the complement has been shown by Kyes³⁰ to consist of lecithin?

Primarily extracts of *Amanita phalloides* dissolve blood corpuscles of various species without the intermediation of blood serum. Thus if corpuscles be washed in physiological salt solution seven or eight times, these corpuscles are still dissolved, and even more readily than are the unwashed corpuscles. Again, if extracts be added to susceptible corpuscles at low temperatures, such as 3° C., hemolysis does not take place. At the end of two hours, if these corpuscles be separated from the supernatant fluid by centrifugation, it is found that this fluid is robbed of its hemolytic properties completely, while the corpuscles, if again suspended in saline solution and placed at 37° C., are dissolved. A number of experiments were carried out, all demonstrating this point. The following experiment is typical:

An extract of *Amanita phalloides* of which $\frac{1}{10}$ c.c. dissolves 1 c.c. of a 5 per cent rabbit-blood suspension in a few minutes was added to washed rabbit corpuscles in equal quantities and placed on ice. At the end of two hours the corpuscles were neither agglutinated nor dissolved. The mixtures of extract and corpuscles were then centrifugalized. The supernatant fluid was no longer hemolytic for rabbit blood, while the corpuscles again suspended in 1 per cent NaCl solution were at once dissolved when placed in the thermostat.

The hemolytic principle of *Amanita phalloides* therefore can unite directly with corpuscles at low temperatures, and can dissolve these corpuscles when freed from all traces of serum, placed at a favorable temperature. In other words, this hemolysin is a single substance acting directly upon susceptible erythrocytes.

Secondarily, the temperature at which the hemolytic activity of *Amanita phalloides* is inhibited is much above that necessary to inactivate serum hemolysins (58.5° C.), and also above that established by Neisser and Wechsberg³¹ for the bacterial hemolysin produced by cultures of *Staph. pyogenes aureus* (56° C.), and for bacterial hemolysins in general. Thus it requires a temperature of at least 60° C. before the extracts lose in hemolytic activity, while this activity is not entirely suspended until the temperature reaches 65° C.

INACTIVATION OF HEMOLYSIN OF *AMANITA PHALLOIDES*.

This is shown in the following table:

TABLE 6.

CONTROL.

EXTRACT IV + 5 PER CENT BLOOD 1.0 C.C.	LYSIS			
	Unheated Extract	Extract heated to 58.5° C. 30 m.	Extract heated to 60° C. 30 m.	Extract heated to 65° C. 30 m.
Extract 1.00 C.C.	Complete	Complete	Complete	None
" 0.1	"	"	Partial	"
" 0.05	"	"	None	"
" 0.01	"	"	"	None
" 0.005	"	"	"	"
" 0.002	"	"	"	"
" 0.001	Trace	Trace	"	None
NaCl sol. only (control)	None	None	"	"

From this table it may be seen that the hemolytic action of *Amanita phalloides* is partially suspended at a temperature of 60° C., but is only completely inhibited at 65° C. Similar experiments were carried out with a great variety of erythrocytes, and, as a general rule, a temperature of 65° C. acting for one-half hour suffices to inactivate this hemolysin for all kinds of blood. Occasionally certain strong extracts must be heated to 70° C. before they cease to exert a trace of hemolytic activity for such very susceptible erythrocytes as those of the guinea-pig.

ATTEMPTED REACTIVATION.

The attempt was made with these inactivated extracts of *Amanita phalloides* to find some substance which would restore to them their hemolytic properties. For this purpose a variety of blood sera, namely, that from guinea-pig, rabbit, fowl, swine, sheep, and beef, as well as cow's milk, were employed. The unheated *Amanita phalloides* was first tested upon rabbit blood to be certain that its activity was still present. It was then heated to 65° C. one-half hour and again tested. If inactive at this point various materials were added for reactivation. Rabbit serum and milk were used undiluted, since no hemolyses of rabbit corpuscles are produced by them. The fowl and swine sera were added only in a dilution of 1:10, quantities greater than this causing a solution of rabbit blood. The following table gives a synopsis of one particular series of reactions:

TABLE 7.
ATTEMPTED REACTIVATION FOR RABBIT BLOOD.

Extract IV (65° C. $\frac{1}{2}$ Hr.)	5 per cent Rabbit Blood	Complemental Substances	Lysis
0.5 c.c.	1 c.c.	1.0 c.c. NaCl 1 per cent	None
0.5	1	1.0 rabbit serum	"
0.5	1	0.1 fowl serum	"
0.5	1	0.3 swine serum	"
0.5	1	0.5 cow's milk	"

From these experiments it may be seen that the substances which are capable of reactivating inactivated serum hemolysins, such as native and foreign sera, are unable to restore to the heated *Amanita phalloides* its hemolytic properties. A large number of different combinations of erythrocytes with different blood sera were made, but in no case did any hemolytic activity manifest itself with the extracts heated to 65° C.

Amanita phalloides, therefore, differs in this respect from the serum hemolysins, but is quite similar to the bacterial hemolysins.

Reactions with resistant erythrocytes.—It has already been pointed out that certain extracts of *Amanita phalloides* exert little or no action upon certain varieties of blood, notably that of beef or sheep. Thus Extract IV, whose hemolytic strength for guinea-pig corpuscles is 0.001 c.c. has practically no action upon sheep blood as may be seen from the following table:

TABLE 8.
REACTION WITH SHEEP BLOOD.

Extract IV + 5% Blood 1.0 c.c.				Lysis
Sheep blood (washed)	+ extract	1.00 c.c.	None
" "	+ "	0.5	"
" "	+ "	0.1	"
" "	+ "	0.05	"
" "	+ "	0.01	"
" "	+ NaCl Sol. only (control)		"
Guinea-pig blood	+ extract	0.001 c.c.	Complete
" " "	+ NaCl sol. only		None

The question comes up whether various sera can furnish the ingredients necessary to bring about complete solution of resistant bloods with extracts of *Amanita phalloides*. A number of reactions were carried out to demonstrate this point, using beef and sheep blood to which extracts of *Amanita phalloides*, active for other corpuscles, were added in large amounts, together with appropriate

quantities of sera. These sera were first tested upon the corpuscles in question to determine the amounts which could be employed with impunity. In the following table, which represents the reactions with sheep blood, fowl, rabbit, and guinea-pig sera were added in 0.05 c.c. quantities only.

TABLE 9.
ATTEMPTED ACTIVATION OF *Amanita phalloides* FOR SHEEP BLOOD.

Extract IV	5 per cent Sheep Blood	Activating Material	Lysis
0.1 c.c.	1 c.c.	1.00 c.c. NaCl 1 per cent	None
0.1	1	1.00 sheep serum	"
0.1	1	0.05 fowl serum	"
0.1	1	0.05 rabbit serum	"
0.1	1	0.05 guinea-pig serum	"

A number of similar combinations of *Amanita phalloides* extract with other varieties of resistant bloods and a large assortment of native and foreign sera were made, but in no case did hemolysis result. The substances necessary for the solution of blood corpuscles resistant to extracts of this fungus are not furnished by blood serum.

Reactions with heated sera.—It has been shown by Calmette³² that certain sera heated to 62.5° C. liberate substances which may act as complements for hemolytic amboceptors.

This reaction has been explained by Kyes as due to the liberation of lecithin, but in view of the possibility that other materials than lecithin might be present in heated serum of a complemental nature it was determined to employ the serum primarily. Beef and swine sera were heated to 65° C. and to 80° C. for thirty minutes, to prevent coagulation the serum being diluted with salt solution. Appropriate quantities were added to mixtures of phalloides extract and resistant corpuscles of beef, sheep, and swine, but in no case was lysis produced. The same sera, when added to inactivated extracts and susceptible erythrocytes of rabbits and guinea-pigs, was without effect upon them. The heating of serum, therefore, does not liberate complement for the hemolysin of *Amanita phalloides*.

Reactions with endocomplement.—The valuable work by Kyes³⁰ in demonstrating that cobra venom can be made to dissolve resistant blood corpuscles by the addition of extracts made from

susceptible corpuscles, the so-called "endocomplement," and in later proving that lecithin is the ingredient of the susceptible corpuscles which plays the part of a complement, has opened up an entirely new field in the study of hemolytic reagents. Kyes's work has been followed by a number of important observations upon other hemolytic substances, notably that of Sachs,³³ who has proved that the laking of blood by sublimate is hastened and increased by lecithin, and that of Pascucci,³⁴ who has demonstrated that ricin, which outside the body merely agglutinates the blood corpuscles, will dissolve them on the addition of lecithin. It is therefore necessary to determine whether endocomplement made from blood corpuscles susceptible to *Amanita phalloides* can have any influence in activating extracts of this fungus for relatively resistant corpuscles or in reactivating these extracts rendered inactive by heat. For this purpose "endocomplement" was prepared from susceptible corpuscles, rabbit and guinea-pig, by washing these corpuscles carefully, laking them with distilled water, and then adding concentrated salt solution in sufficient quantity to bring the solution up to the requisite tonicity. In a number of reactions this endocomplement was employed for activation and for reactivation, but in all cases the results were negative, as can be seen below.

TABLE II.
REACTIONS WITH ENDOCOMPLEMENT.
CONTROL WITH *Amanita phalloides*.

EXTRACT K	LYSIS				
	5 per cent Rabbit Blood 1.0 c.c.	5 per cent Beef Blood 1.0 c.c.	5 per cent Beef Blood 1.0 c.c. + Rabbit Endocom- plement 1.0 c.c.	5 per cent Sheep Blood 1.0 c.c.	5 per cent Sheep Blood 1.0 c.c. + Rabbit Endocom- plement 1.0 c.c.
1.00 C.C.	Complete	None	None	None	None
0.25	"	"	"	"	"
0.10	"	"	"	"	"
NaCl sol. only (control)	None	"	"	"	"

Endocomplement was also employed in a number of other ways for activating *Amanita phalloides* for resistant corpuscles and for reactivating extracts of the fungus heated to 65° C.; in all cases the extracts of the susceptible corpuscles had no influence in determining the solution of blood corpuscles.

Reactions with lecithin.—Lecithin obtained from Merck, dissolved

in methyl alcohol, in a 1 per cent solution, then diluted with physiological salt solution, was tested to determine whether it enters into combination with *Amanita phalloides*. These experiments were conducted mainly with beef, sheep, and swine blood, although rabbit and guinea-pig corpuscles were also tested with inactivated *Amanita phalloides* and lecithin. A considerable number of reactions were carried out, in all of which the results were negative. It is necessary, therefore, to give only one or two examples, which are represented in the following table:

TABLE 12.
ACTIVATION WITH LECITHIN.
CONTROL.

Amanita extract was first tested upon 5 per cent rabbit-blood suspension and found to be active in a dilution of 1:100. The undiluted material was inactive for beef blood. Various dilutions of lecithin dissolved in methyl alcohol (1 per cent solution) were tested upon beef blood and found to be inactive in 0.003 c.c. quantities.

Lecithin was now employed in 0.003 c.c. quantities for the activation of *Amanita phalloides* for beef blood.

Extract IV	5 per cent Beef Blood (Washed)	Lecithin, 1 per cent	Lysis
1.0 c.c.	1 c.c.	0.003 c.c.	None
0.1	1	0.003	"
0.05	1	0.003	"
0.01	1	0.003	"
NaCl sol. only (Control)	1	0.003	"

Here it may be seen that lecithin has no action in causing a solution of resistant corpuscles by means of inactive extracts of *Amanita phalloides*. The ingredients of the mixtures were varied, using different quantities of *Amanita phalloides* and various amounts of lecithin, as well as of other fairly resistant corpuscles; but it could not be determined that lecithin exerted any influence in the reactions. Lecithin was also employed for reactivating the heated extracts, but the results were negative.

TABLE 13.
REACTIVATION OF INACTIVATED *Amanita phalloides* WITH LECITHIN.

Extract IV was first tested upon rabbit blood and then inactivated at 65° C. Lecithin was tested upon rabbit corpuscles and found to give a solution of them in quantities greater than 0.003 c.c. This amount of lecithin was therefore employed for reactivation.

Extract IV, 65° C. Half Hour	5 per cent Rabbit Blood (Washed)	Lecithin, 1 per cent	Lysis
1.0 c.c.	1 c.c.	0.003 c.c.	None
0.1	1	0.003	"
0.05	1	0.003	"
0.01	1	0.003	"
NaCl sol. only (Control)	1	0.003	"

COMBINATIONS OF RESISTANT CORPUSCLES WITH *AMANITA PHALLOIDES*.

The question arises whether the failure of certain resistant corpuscles like those of beef, sheep, or swine to dissolve, when combined with certain extracts of *Amanita phalloides* which act vigorously upon other erythrocytes, is due to the lack of certain ingredients in the corpuscles themselves, necessary for hemolysis, or is the result of the inability of these resistant corpuscles to unite with the hemolytic principles. Such a question, from its very nature, is not capable of exact answer. It may be answered approximately by determining whether the extracts of *Amanita phalloides* lose in hemolytic strength for susceptible corpuscles if treated with the more resistant erythrocytes. Sheep corpuscles were combined with extracts of *Amanita phalloides*, which had a definite strength for guinea-pig blood, and after the lapse of sufficient time for hemolytic reaction the corpuscles were removed by centrifugation, and the supernatant fluid again tested for hemolytic activity for guinea-pig blood. These reactions are given in the following table:

TABLE 14.
ABSORPTIVE POWER OF SHEEP BLOOD.

Amanita phalloides extract was first tested upon guinea-pig corpuscles (washed) and found to give a complete solution in 0.003 c.c. quantities. Appropriate quantities of the extract were now combined with sheep blood which is not dissolved.

Extract VI	5 per cent Sheep Blood (Washed)	Lysis
1.0 C.C.	1 C.C.	None
0.8 1	1	"
0.5 1	1	"
0.3 1	1	"
0.1 1	1	"
NaCl sol. only (Control).....	1	"

These tubes were now centrifugalized and the supernatant fluid tested for hemolytic strength for guinea-pig corpuscles. The strength for these corpuscles is unaltered.

Extract VI (from Sheep Blood)	5 per cent Guinea-Pig Blood (Washed)	Lysis
0.25 C.C.	1 C.C.	Complete
0.10 1	1	"
0.01 1	1	"
0.008 1	1	"
0.005 1	1	"
0.003 1	1	"
0.001 1	1	Partial
NaCl sol. only (Control)	1	None

Therefore resistant corpuscles can remove from extracts of *Amanita phalloides* little or none of the hemolytic material which is capable of acting upon susceptible blood.

In the same way extracts of *Amanita phalloides* can be combined with the stroma of resistant corpuscles with practically no change in their hemolytic strength for susceptible erythrocytes.

Thus 2 c.c. of Extract V which gives a complete solution of rabbit corpuscles in 0.003 c.c. quantities was combined with the stroma from 20 c.c. washed beef blood. After 18 hours the supernatant fluid was tested for hemolytic strength for rabbit blood.

TABLE 15.
ABSORPTIVE POWER OF STROMA.

Fluid from Stroma	5 per cent Rabbit Blood	Lysis
0.10 C.C.....	1 C.C.	Complete
0.05	1	"
0.01	1	"
0.008	1	"
0.005	1	"
0.003	1	"
0.002	1	None
0.001	1	"
Control 1 C.C.....NaCl 1%	1	"

It is evident that the original strength for rabbit blood 0.003 c.c. is maintained in the supernatant fluid.

From these experiments it may be seen that the failure of the resistant corpuscles to dissolve is in all probability due to the failure of the corpuscles to unite with hemolytic ingredients of the extracts, unless another possibility be suggested, that there are multiple hemolysins present in *Amanita phalloides* each acting upon a special variety or group of erythrocytes. This latter question has not been definitely settled. It has been found by experimentation with more concentrated extracts of the fungus, and a greater variety of beef, sheep, and swine bloods, that these corpuscles exhibit great differences in susceptibility at different times, especially if not freed from all traces of serum. Eventually extracts of *Amanita phalloides* were obtained which completely dissolved all varieties of corpuscles, but not, however, in great dilution.

These reactions can be seen in the following table:

TABLE 16.

SYNOPSIS EXTRACT V.

Extract V Prepared from about 50 Grams *Amanita phalloides* and 200 c.c. Distilled Water.
STRENGTH FOR VARIOUS ERYTHROCYTES.

Guinea-pig	0.002 c.c.
Rabbit	0.003
Beef	0.5
Sheep	1.0

We have thus been able to confirm Kobert's observations upon the solvent action exerted upon beef blood. We have never been able to obtain a solution of these corpuscles in such a great dilution as did Kobert, but he employed relatively enormous quantities of the fungus, 1,000 grams, and evaporated the extracts to dryness. Our own purified hemolysins evaporated to dryness have not been tested upon beef blood.

REACTIONS WITH LYMPHOCYTES.

The great susceptibility of red blood corpuscles to extracts of *Amanita phalloides* suggested the possibility of a definite leucolytic action. To test this, lymphocytes were employed, the mesenteric lymphatic glands from rabbits being emulsified with salt solution. Appropriate quantities of this emulsion were combined with extracts of *Amanita phalloides* in various ways but without effect.

1 c.c. extract + 1 c.c. lymphocyte emulsion	No lysis
1 " " heated to 65° C. + 1 c.c. lymphocyte emulsion	" "
1 " " " 80° C. + 1 " " " "	" "
Control 1 c.c. NaCl 1 per cent + 1 " " " "	" "

There is, therefore, no definite solvent action upon lymphocytes outside the animal body. This experiment does not of course indicate that there may not be definite necrosis of the cells by the prolonged action of *Amanita phalloides* in the tissues.

NATURAL ANTIHEMOLYSINS.

In view of the great desirability of obtaining remedies which can be employed in the immediate treatment of poisoning by *Amanita phalloides*, a number of experiments were undertaken to determine whether any substances exert an antagonistic or an anti-hemolytic action upon extracts of this fungus. In these experiments native and foreign blood sera, raw and heated, raw and boiled milk and lecithin, were utilized. The extracts were first tested upon such susceptible corpuscles as those from guinea-pigs and rabbits,

which were dissolved in 0.005 c.c. quantities. Guinea-pig blood also was dissolved in 0.001 c.c. quantities and rabbit blood partially. Rabbit and guinea-pig sera were added undiluted for their homologous erythrocytes. Raw and boiled milk were employed in any desirable quantity since no hemolysins are present in this substance, at least for the corpuscles in question. Lecithin was used only in 0.003 c.c. quantities. The following table gives a synopsis of the results obtained:

TABLE 17.
NATURAL ANTIHEMOLYSINS.

Extract IV	5 per cent Blood (Washed) 1 c.c.	Antilysins	Lysis
0.005 c.c.	Guinea-pig blood		Complete
0.001	" "		"
0.005	" "	1.000 c.c. guinea-pig serum	"
0.001	" "	1.000 "	None
0.005	" "	1.000 milk	"
0.001	" "	1.000 "	"
0.005	" "	0.003 lecithin	Complete
0.001	" "	0.003 "	"
0.005	Rabbit blood		Complete
0.001	" "		Trace
0.005	" "	0.5 c.c. raw milk	None
0.001	" "	0.5 "	"
0.001	" "	1 boiled milk	Partial
0.005	" "	1 " "	None
0.001	" "	1 " "	"
0.005	" "	1 rabbit serum	"
0.001	" "	1 " "	"
0.005	" "	1 " " heated to 65° C.	"
0.001	" "	1 " " " 65° C.	"

The influence of other sera, fowl, sheep, beef, and swine, upon the hemolytic activities of *Amanita phalloides* was also tested with rabbit and guinea-pig corpuscles. In all cases the foreign and native serum, exhibited a definite antihemolytic action. The strongest of all substances in this respect proved to be milk, both raw and boiled; and milk may be regarded as a mild natural antidote for *Amanita phalloides*.

REACTIONS WITH CHOLESTERIN.

The discovery of Ransom,³⁵ working in Meyer's laboratory, that the constituent of the corpuscular stroma which is attacked by saponin, and the ingredient of the blood serum which neutralizes it, is cholesterin, rendered it imperative to determine whether the antihemolytic action exerted upon extracts of *Amanita phalloides* by blood serum and milk can be due to the same substance.

To demonstrate this point, cholesterol was dissolved in ether, in a 1 per cent solution of which 1 c.c. was added to 2 c.c. of phalloides extract. As a control 1 c.c. of ether was added to another tube of the extract and both preparations were placed in the thermostat for three hours. At the end of this time the ether had evaporated and cholesterol crystals had begun to deposit in the cholesterol-phalloides mixture. The two mixtures were now tested for their hemolytic strength upon rabbit corpuscles, at the same time the crude phalloides extract being tested by way of comparison. The results are given below:

TABLE 19.
ACTION OF CHOLESTERIN UPON
HEMOLYSIS PRODUCED BY *Amanita phalloides* IN 5 PER CENT RABBIT BLOOD.

Quantities	Cholesterin Mixture	Other Mixtures	Crude Material
1.0 c.c.	Complete	Complete	Complete
0.1	"	"	"
0.05	"	"	"
0.01	"	"	"
0.0075	None	None	None
0.005	"	"	"
0.001	"	"	"

CONTROL.—1 c.c. NaCl 1 per cent+1 c.c. 5 per cent rabbit blood=None.

Hemolysis is produced in rabbit blood by the various mixtures in exactly the same quantity, and cholesterol cannot be considered the ingredient of the serum which exerts this antagonistic action upon *Amanita phalloides*. This action is in all probability due to the albuminous constituents of serum and milk.

SUMMARY OF HEMOLYTIC REACTIONS.

From these experiments it may be seen that extracts of *Amanita phalloides* contain a hemolytic principle which has been isolated by Kobert to which he gave the name "phallin." This substance dissolves all varieties of erythrocytes which exhibit great differences in susceptibility. It enters into direct combination with the red blood cells, dissolving them without the intermediation of serum. It is inactivated at a temperature of 65° C. acting for one-half hour; this inactivation is apparently a destruction, since all substances capable of reactivating serum hemolysins, including serum, corpuscular extracts, and lecithin, fail to restore to this heated material its hemolytic properties. Its activity is inhibited by blood sera and by raw and boiled milk, the latter of which may be looked upon as a natural antidote of mild strength. In virtue of these properties phallin should be provisionally classed with the bacterial hemolysins.

4. ACTION OF *AMANITA PHALLOIDES* UPON ANIMALS.

If we inoculate small animals such as rabbits or guinea-pigs with minute quantities of extracts made from *Amanita phalloides* it is found that this substance is possessed of extreme toxicity. Thus in an extract prepared from 8 grams of dried *Amanita phalloides* and 100 c.c. of distilled water, 1 c.c. will kill a rabbit of 2,000 gram weight in 48 hours, and 0.5 c.c. will kill a guinea-pig of 500 gram weight in the same time. Within a few hours after the inoculation the animals show signs of poisoning, slinking to a corner of the cage with ruffled fur and refusing to eat. They rapidly lose weight and strength, when placed on their back or sides being unable to get to their feet. After a period of time varying from 24 hours to three days they develop great difficulty in breathing, the respirations being much diminished in frequency and increased in depth. The respirations gradually cease, while the heart continues to beat forcibly for some seconds after the respirations are no longer visible. If the chest wall be opened immediately, the heart may be found to be still beating.

With small, feeble guinea-pigs death may occur within a few hours after subcutaneous inoculation, but with normally healthy guinea-pigs and rabbits the period of illness lasts usually one to six days.

Small doses induce a chronic intoxication, especially in rabbits, which die at the end of three, four, or even six weeks. Guinea-pigs, if they survive 12 to 15 days, practically never die of the late changes. Convulsions or convulsive movements do not occur ordinarily, although convulsions may be seen as a rare terminal event. Salivation and gastro-intestinal symptoms are never present and phalloides poisoning may thus be clearly differentiated from muscarin poisoning.*

Postmortem changes.—The lesions following subcutaneous inoculation are definite and characteristic. There is seldom postmortem rigidity. At the site of inoculation there is a most extensive subcutaneous edema, with numerous small areas of hemorrhage scattered about in the edematous tissue and in the adjacent muscles. The subcutaneous lymphatic glands are swollen and hemorrhagic. The serous membranes are free from excess of fluid, but there may be

**Amanita phalloides* has no action upon the cornea, such as that shown by ricin.

hemorrhages in the mesentery and occasionally in the pleura. The adrenals are always hemorrhagic, and there may be hemorrhages in the mesenteric lymphatic glands. The liver and kidneys occasionally show hemorrhagic areas on their surfaces, and there are always minute areas of hemorrhage in their interior. The bladder is filled with urine which may be blood stained. The spleen is enlarged and soft. The lungs may show small hemorrhages, which may also rarely be present on the pericardial surface of the heart. This organ is always greatly dilated, the contained blood being fluid or rarely clotted. The blood in the larger vessels is occasionally fluid, and in the acute cases is much darker in color than is the normal blood. The stomach contents are frequently blood stained, and minute ulcers are found in the submucosa with small hemorrhages at their bases. Both brain and spinal cord also show definite areas of hemorrhage.

With small doses, when the animals die of chronic intoxication, the lymphatic glands and adrenals are usually hemorrhagic, but the most marked changes are the extreme fatty degeneration and the necrosis of the liver and other organs.

Microscopic examination.—Microscopically the changes seen are similar to those already described. The evidence of hemorrhage in lymphatic glands, in liver, spleen, and kidneys is well marked. There are countless red blood corpuscles scattered through the tissues, and occasionally degeneration of the endothelial lining of the blood vessels may be discerned. An extensive deposition of blood pigment has taken place in the spleen. The muscle fibers of the heart show hyaline degeneration, and minute areas of necrosis with small-celled infiltration are occasionally found. There is an extensive deposition of fat in various regions, but most marked in the liver, and necrotic cells may be found in nearly all the organs.

The following protocol is a fair example of the effects of subcutaneous inoculation:

Guinea-pig weighing 715 grams inoculated January 20, 1905, with 1 c.c. of an extract of *Amanita phalloides* made from 8 grams dried material and 100 c.c. distilled water. The following day the animal was found in the corner of the cage with ruffled fur and refusing its food. It gradually developed great weakness. When placed on its side it got to its feet only with difficulty. There was no salivation, no convulsive movements, no diarrhea. The respirations were much lessened

in frequency and increased in depth. Gradually increasing dyspnea was followed by death at 4 P. M., January 24, four days after inoculation.

Autopsy was held at once. An enormous gelatinous edema was present over the entire anterior abdominal wall with small hemorrhages present in this edematous tissue. Subcutaneous lymphatic glands swollen and hemorrhagic. Peritoneal cavity free from exudate. Vessels of mesentery injected. Spleen congested, dark, and friable. Liver surface shows several small hemorrhages and several opaque areas of fatty degeneration. Adrenals hemorrhagic; kidneys apparently normal; bladder filled with blood-stained urine; pleural cavity free from fluid; lungs show a few small areas of hemorrhage; heart much dilated; blood fluid, but clots on exposure to the air; brain and spinal cord also show hemorrhagic areas.

Cultures from heart, liver, and peritoneal cavity were sterile.

ESTIMATION OF MINIMUM FATAL DOSES.

By inoculating a considerable series of animals with graduated doses of phalloides extracts it is possible to determine, within narrow limits, the quantity which will kill animals of a definite weight. Two different limits of toxicity may be made out, the smallest quantity which will kill an animal acutely and the smallest quantity which will kill an animal by chronic intoxication. Then by inoculating another series of animals with a quantity somewhat in excess of the dosage already estimated, that amount of *Amanita phalloides* which will always kill animals of a definite weight in a definite time may be determined. This quantity of any extract may then be considered the M. L. D. of that extract. By comparing the minimum fatal dose with the hemolytic strength, we obtain a ratio of toxicity to hemolysis which gives us a rough guide for estimating the toxicity of new solutions. With extracts freshly made from the dried fungus, this ratio of toxicity to hemolysis is fairly constant, and by making two or three control inoculations to discover whether the poisons have degenerated we are in possession of sufficient data in regard to any particular extract to guide us in experimental work. A considerable number of different extracts of the fungus were compared for hemolytic strength and for toxicity upon both rabbits and guinea-pigs, and it was learned that not only is the quantity of hemolysin present a fairly accurate guide to the toxicity, but that both hemolytic activity and toxicity suffer little impairment from the passage of time.

The toxicity of Extract I, whose hemolytic reactions have already been carefully considered, is given in the following table:

TABLE 20.
TOXICITY OF EXTRACT I FOR RABBITS.

Weight in Grams	Dosage	Interval before Death
1,220.....	2.0 c.c.	48 hours
850.....	1.5	24 "
870.....	0.5	48 "
2,000.....	0.3	14 days
1,850.....	0.2	6 "
Minimum fatal dose for rabbits 2,000 grams 0.3 c.c.		
" " " " " 1,500 " 0.2		
Dose sufficient to cause acute death in rabbit of 1,000 grams 0.5		
Hemolytic strength for rabbit blood 0.05		

TOXICITY OF EXTRACT I FOR GUINEA-PIGS.

Weight in Grams	Dosage	Result
552.....	2.0 c.c.	Death in 3 days
715.....	1.0	" " 4 "
455.....	1.5	" " 1 day
250.....	0.2	" " 1 "
240.....	0.1	" " 1 "
437.....	0.1	Recovery
305.....	0.075	"
427.....	0.05	"
Minimum fatal dose for guinea-pig 500 grams 0.5 c.c.		
" " " " " 250 " 0.2		
Hemolysis for guinea-pig blood 0.005		

The strength of other extracts was also tested upon animals. Thus 0.34 c.c. of Extract IV made from 15 grams dried *Amanita phalloides* and 200 c.c. distilled water, whose hemolytic strength for rabbit blood was 0.002 c.c., killed a rabbit of 847 grams in less than 24 hours, with the usual appearances of gelatinous edema, hemorrhages, and degenerations.

Again the toxicity of Extract V prepared from 30 grams dried *Amanita phalloides*, and 200 c.c. distilled water, whose hemolytic strength for guinea-pig blood was 0.002 c.c., was tested upon guinea-pigs with the following result:

Weight in Grams	Dosage	Result
579.....	1.0 c.c.	Death in 3 days
499.....	0.5	" " 24 hours
429.....	0.25	" " 24 "

Here it may be seen that the toxicity of Extract V is somewhat greater than that of Extract I, 0.25 c.c. sufficing to kill a guinea-pig of 427 gram weight in 24 hours.

5. IMMUNIZATION OF ANIMALS.

If we treat animals with gradually increasing doses of *Amanita phalloides* it is possible to accustom them to the material, provided the initial dose be much under that capable of causing a chronic intoxication. This immunization must be carried out over a period of many weeks, as the rapid repetition of the doses, and the increase in their amount causes a high mortality. By treating a number of rabbits with various quantities of *Amanita phalloides* extracts, at various intervals of time eventually the proper method of successfully immunizing them was worked out, and a number of rabbits were immunized to the point where they would withstand the inoculation of rapidly-fatal doses, and several multiples of fatal doses.

The following table gives a synopsis of the treatment of one of the successfully immunized rabbits:

TABLE 21.		
IMMUNE RABBIT A, WEIGHT 2,020 GRAMS.		
Immunized to Extract IV whose Hemolytic Strength for Rabbit Blood is 0.002 c.c.		
Date of Inoculation	Quantity given	
February 9	0.002 c.c.
" 11	0.004
" 13	0.008
" 15	0.010
" 20	0.032
" 25	0.064
March 6	0.14
" 11	0.25 1 fatal dose
" 17	0.4
" 24	0.6 2 fatal doses
" 31	0.8
April 7	1.0 3-4 fatal doses
Bled April 14, 20 c.c.		

ANTIHEMOLYTIC PROPERTIES OF IMMUNE SERA.

At this point the animals were bled and their sera subjected to a careful study upon blood corpuscles and upon animals. Primarily such blood sera is *antihemolytic*. It completely inhibits the solution of blood corpuscles by *Amanita phalloides* and in considerable dilutions. If we estimate the smallest quantity of any extract which will completely dissolve 1 c.c. of a 5 per cent suspension of guinea-pig or rabbit blood corpuscles, the immune serum from Rabbit A completely arrests this solution of corpuscles in a dilution of 1:1,000. Thus the serum from Rabbit A was tested for anti-

hemolytic properties on April 15 with the results shown in the following table:

TABLE 22.
ANTIHEMOLYTIC STRENGTH OF SERUM RABBIT A.
CONTROL *Amanita phalloides* EXTRACT.

Extract V+5 per cent Rabbit Corpuscles 1.0 c.c. + Immune Serum							Lysis
Extract V	0.005 c.c.	+ 1.0 c.c. NaCl 1%	Complete
"	0.003	+ 1.0	"	I	.	.	"
"	0.002	+ 1.0	"	I	.	.	Partial
"	0.001	+ 1.0	"	I	.	.	Trace
"	0.005	+ normal serum	1.0 c.c.	.	.	.	Complete
"	0.005	+ immune	"	1.0	.	.	None
"	0.005	+ "	"	0.01	.	.	"
"	0.005	+ "	"	0.0075	.	.	Faint trace
"	0.005	+ "	"	0.001	.	.	"
"	0.005	+ "	"	0.0001	.	.	Partial
"	0.003	+ normal	"	0.1	.	.	Complete
"	0.003	+ immune	"	0.1	.	.	None
"	0.003	+ "	"	0.01	.	.	"
"	0.003	+ "	"	0.0075	.	.	"
"	0.003	+ "	"	0.001	.	.	"
"	0.003	+ "	"	0.0001	.	.	Complete
NaCl sol. only (control)	None

The serum from Rabbit A therefore has an antihemolytic strength for rabbit corpuscles of 1:1000.

The same serum was tested upon guinea-pig corpuscles with similar results.

TABLE 23.
ANTIHEMOLYTIC STRENGTH FOR GUINEA-PIG BLOOD.
CONTROL *Amanita phalloides* EXTRACT.

Extract V+5 per cent Guinea-Pig Corpuscles 1.0 c.c. + Immune Serum							Lysis
Extract V	0.005 c.c.	+ 1.0 c.c. NaCl sol. 1%	Complete
"	0.003	+ 1.0	"	"	I	.	"
"	0.002	+ 1.0	"	"	I	.	"
"	0.001	+ 1.0	"	"	I	.	Partial
"	0.002	+ normal serum	1.0 c.c.	.	.	.	Complete
"	0.002	+ immune	"	1.0	.	.	None
"	0.002	+ "	"	0.1	.	.	"
"	0.002	+ "	"	0.01	.	.	"
"	0.002	+ "	"	0.0075	.	.	"
"	0.002	+ "	"	0.001	.	.	"
"	0.002	+ "	"	0.0001	.	.	Partial
NaCl sol. only (control)	None

Therefore, the serum of Rabbit A has an antihemolytic action upon guinea-pig corpuscles in a dilution of 1:1000.

ANTITOXIC PROPERTIES OF IMMUNE SERA.

The blood serum of these immune animals besides possessing antihemolytic properties is definitely *antitoxic*. If doses of *Amanita phalloides* much in excess of minimum fatal doses be mixed with appropriate amounts of the immune serum, they can then be administered to susceptible animals without deleterious effects. Such antitoxic properties are shown in the following table:

TABLE 24.

ANTITOXIC ACTION OF IMMUNE SERUM A.

CONTROL.—Rabbit weighing 2,000 grams inoculated with 1 c.c. of Extract V. Death in 48 hours with typical lesions.

Serum tested upon seven rabbits, all well under 2,000 grams weight.

Dosage	Amount Immune Serum	Result
1 c.c.	3.0 c.c.	No edema site of inoculation
1	2.0	" " " " "
1	1.5	" " " " "
1	1.0	Edema site of inoculation
1	0.5	" " " " "
1	0.25	" " " " "
1	0.125	" " " " "

All of these animals were watched for a period of 10 weeks, during which the edema at the site of inoculation disappeared and the animals remained free from any signs of chronic intoxication. Quantities of antiserum from Rabbit A above 1 c.c. completely neutralize three to four times a fatal dose of *Amanita phalloides* for rabbits of 2,000 grams, and quantities less than 1 c.c. save the animals from death. The same serum possesses antitoxic properties for guinea-pigs.

A number of rabbits were successfully immunized to the same point as was Rabbit A, but to be absolutely certain that one is dealing with a definite antitoxin, it is necessary to produce a higher degree of resistance and to obtain a serum which will neutralize larger multiples of fatal doses. With small animals it is not possible to produce antitoxic sera of higher potency, but it seemed desirable to obtain one in which 1 c.c. would neutralize at least 10 times a fatal dose for a susceptible animal, the standard originally set up by Ehrlich upon von Behring's work, as a *normal serum*. Many difficulties were encountered when the attempt was made to make rabbits resistant to large doses of *Amanita phalloides*, as these animals were completely overwhelmed by the excessive doses and frequently died acutely. Eventually by beginning the immunization with subcuta-

neous inoculations and following this intraperitoneal inoculation of the larger quantities several animals were successfully handled.

The details of the immunization of one of these animals are given in the following table:

TABLE 25.			
IMMUNE RABBIT C.			
IMMUNIZED TO EXTRACT IV. WEIGHT 2,000 GRAMS.			
Date		Dosage	
February	9	0.002 c.c.	
"	14	0.004	
"	20	0.008	
"	25	0.016	
March	5	0.032	
"	9	0.064	
"	14	0.14	
"	18	0.25	fatal dose
"	24	0.4	
"	31	0.6	2 fatal doses
April	7	0.8	
"	14	1.0	3-4 fatal doses
May	1	1.0	3-4 " "
May 8, bled 45 c.c.			
May	18	0.75 c.c.	3 fatal doses
"	26	1.0	3-4 " "
June	6	1.25	4 " "
"	19	1.5	4-5 " "
"	28	2.0	6-7 " "

Bled July 6, 1905.

The serum from Rabbit C was tested May 8 after it had been successfully immunized to withstand acutely fatal doses. At this point it had an antihemolytic strength of 1:1,000. Its antitoxic strength was tested only with an excess of *Amanita phalloides* as is shown in the following table:

TABLE 26.	
CONTROL RABBIT INOCULATED WITH 2 c.c. EXTRACT V, REPRESENTING SIX TO SEVEN TIMES A FATAL DOSE.	
DEATH 18 HOURS.	
I. RABBIT.	—Inoculated May 9, 2 c.c. Extract V and 3 c.c. antiserum. Developed some edema at site of inoculation. This disappeared and the animal remained perfectly well for 10 weeks.
II. RABBIT.	—Inoculated May 9, 2 c.c. Extract V and 2 c.c. antiserum. Death in 24 hours.
III. RABBIT.	—Inoculated May 9, 1 c.c. Extract V and 1.2 c.c. antiserum. This animal developed edema at the site of inoculation, but this disappeared completely and the animal remained well.

From this series of animals it may be seen that at the time when the serum from Rabbit C had an antihemolytic strength of 1:1,000

it was possessed of definite antitoxic properties. Thus 2 c.c. of Extract V, representing six to seven times a fatal dose for rabbits of 2,000 grams, is completely neutralized by 3 c.c. of the antiserum, while half that quantity of the extract, representing three to four times a fatal dose, requires a little more than 1 c.c. serum for neutralization. Mixtures of phalloides extract and serum in which the proportion of serum is below this ratio are not neutralized.

At this point, although definite multiples of fatal doses had been neutralized, it was determined to push the animal still further, and the inoculations were continued over another period of two months. Considerable intervals of time were allowed to elapse between the doses, which were given intraperitoneally to avoid the profound local necrosis produced by the subcutaneous inoculation of large quantities of phalloides extract.

This particular animal stood the injections well, finally receiving 2 c.c. of Extract V, representing six to seven times a fatal dose for a rabbit of 2,000 grams. All told the animal received 11 c.c. of this solution, representing about 33 times a fatal dose for a rabbit of its weight. Its serum was finally again withdrawn after the last inoculation and tested for antihemolytic and antitoxic strength. At this time it would prevent hemolysis by *Amanita phalloides* in a dilution of 1:10,000 and 1 c.c. of the serum would neutralize 2 c.c. of Extract V, as can be seen in Table 27:

TABLE 27.

I. RABBIT.—2 c.c. Extract V—2 c.c. Antiserum C—Recovery
II. RABBIT.—2 c.c. Extract V—1 c.c. Antiserum C—Recovery
III. RABBIT.—2 c.c. Extract V—0.5 c.c. Antiserum C—Death
IV. RABBIT.—2 c.c. Extract V—0.25 c.c. Antiserum C—Recovery

The average weight of these rabbits was 1,500 grams. From this table it may be seen that 1 c.c. of the serum of Rabbit C, which has an antihemolytic strength of 1:10,000, possesses also an antitoxic strength of such an amount that 1 c.c. will neutralize six to seven times a fatal dose for a rabbit of 2,000 grams and 10 times a fatal dose for a rabbit of 1,500 grams. Such a serum may roughly be compared with an antitoxic serum of low potency in that 1 c.c. neutralizes 10 minimum fatal doses.

CELLULAR IMMUNITY.

The question arises, in the consideration of several problems of immunity, whether the cells of the body acquire any resistance to the action of toxins in immunization, or whether protection from fatal doses is due to the antitoxic properties which the surrounding blood serum possesses. With a hemolytic agent, such as phalloides extract, which acts so vigorously upon the erythrocytes this question is easily settled for these particular cells.

Blood from Rabbit C, which was completely immunized to *Amanita phalloides*, was withdrawn and tested in the usual manner. This blood in 5 per cent suspension was tested without washing and after being washed five times. The results may be seen in the following table:

TABLE 28.
CELLULAR IMMUNITY.
CONTROL *Amanita phalloides* EXTRACT.

EXTRACT V +5 PER CENT RABBIT BLOOD 1.0 C.C.	LYSIS		
	Normal Rabbit Blood	Blood Rabbit C Unwashed	Blood Rabbit C Washed 5 Hours
Extract 0.10 c.c.....	Complete	Complete	Complete
" 0.04	"	None	"
" 0.02	"	"	"
" 0.01	"	"	"
" 0.005	Partial	"	Partial
NaCl sol. only (control).....	None	"	None

From these observations it is apparent that, whereas the unwashed corpuscles of the immune rabbit exert considerable resistance to hemolysis by *Amanita phalloides*, the corpuscles deprived of their serum and suspended in normal saline solution are susceptible to the same extent as are the normal corpuscles of the same species. The resistance of the corpuscles to solution by the hemolysin is therefore due to the antihemolytic properties of the blood serum in which the corpuscles are suspended, the corpuscles themselves possessing no increased resistance. There is therefore *no cellular immunity* possessed by the erythrocytes of animals highly immunized to *Amanita phalloides*.

6. CONCLUSIONS.

In consideration of the results obtained from the investigations reported in this paper, it may be stated that the poisons of *Amanita*

phalloides, of which the phallin of Kobert is the hemolytic constituent, are of the nature of toxins in that they act upon the animal body after a definite latent period, produce lesions which are characteristic of bacterial intoxications in general, and produce immunity in susceptible animals by the inoculation of non-lethal doses. Such immunized animals will eventually withstand the inoculation of considerable multiples of fatal doses and their blood serum possesses antihemolytic and antitoxic properties, a serum eventually being obtained in which 1 c.c. will neutralize 10 times a fatal dose of the poison. Whether sera of greater potency can be produced by the treatment of larger animals is a problem for the future to decide, but in favor of the attempt to produce sera which can be used for curative purposes in man may be mentioned the following facts: The number of cases of fungus poisoning is numerous, far more so than is generally recognized, and the vast majority of all deaths from this cause are due to a single species, *Amanita phalloides*; the mortality is high, varying from 60 to 100 per cent; the clinical symptoms are characteristic, the suffering is extreme, and the diagnosis is easily made from the history; the physician is called to the case almost at once, and a period of six to eight days in adults and three to four days in children elapses between the time of poisoning and the fatal outcome, during which curative sera could be administered.

7. BIBLIOGRAPHY.

1. PAULET. *Traité des champignons*. Paris, 1793.
2. BARDY. *Bull. Soc. philomat. des Vosges*, 1883-84, 9.
3. GUILLAUD. *Bull. Soc. mycolog. de France*, 1885, 1, p. 123.
4. INOKO. *Mitth. a. d. med. Fac. d. k. Jap. Univ.*, Tokio, 1887-89, 1, p. 227-306.
5. FALCK. *Handbuch der gesammten Arzneimittellehre mit Einschluss der Toxicologie*, I, 12, p. 282.
6. PALMER. Quoted by Forster in the *Medical Communications*, Mass. Med. Soc., 1890, 15, p. 209.
7. FORSTER. *Boston Med. and Surg. Jour.*, 1890, 125, p. 267.
8. PFROMM. *Med. Bull. Phila.*, 1905, 27, p. 401.
9. PLOWRIGHT. *Brit. Med. Jour.*, 1905, 2, p. 541.
10. SCHMIEDEBERG UND KOPPE. *Das Muscarin*. Leipzig, 1869.
11. HARNACK. *Arch. j. exp. Path. u. Pharm.*, 4, p. 168; 6, p. 101.
12. PRENTISS. *Phila. Med. Jour.*, 1898, 2, p. 607.
13. BOEHM UND KÜLZ. *Arch. j. exp. Path. u. Pharm.*, 1885, 19, p. 403.
14. BULLIARD. *Histoire des champignons de France, 1791-1812*.

15. PLOWRIGHT. *Lancet*, 1879, 2, p. 941.
16. GILLOT. *Etude medicale sur l'empoisonnement par les champignons*. Lyons. 1900.
17. MASCHKA. *Prag. Vierteljahrsschrift*, 1855, 2.
18. STUDER SAHLI UND SCHÄRER. *Mitth. d. naturf. Gesellsch. in Bern*, 1884-85, p. 77.
19. TAPPEINER. *München. med. Wchnschr.*, 1895, 42, p. 133.
20. MOERS. *Ztsch. f. Med.-Beamte*, 1903, 16, p. 412.
21. BOUDIER. *Die Pilze in ökonomischer chemischer u. toxicologischer Hinsicht*, 1868.
German translation of French original.
22. LETELIER ET SPENEUX. *Ann. d'hyg. publ.*, 1867, p. 71.
23. ORÉ. *Bull. Acad. de méd.*, 1877, 13, p. 350; 32, p. 877; *Arch. de physiol. norm. et path.*, 1877, 2, p. 275.
24. KOBERT. *St. Petersburger med. Wchnschr.*, 1891, 16, pp. 463, 471.
25. SEIBERT. Inaug. Dissert. München, 1893.
26. ATKINSON. *Mushrooms*. New York, 1903.
27. FARLOW. "Some edible and poisonous fungi." *Bulletin No. 15*, 1898, U. S. Dept. of Agric., Division of Vegetable Physiology and Pathology.
28. PECK. *Various Annual Reports of the Regents of the State of New York*, Albany, N. Y.
29. FLEXNER AND NOGUCHI. *Jour. Exp. Med.*, 1901-5, 6, p. 277.
30. KYES. *Berl. klin. Wchnschr.*, 1902, pp. 886, 918; 1903, pp. 956, 882; 1904, p. 494.
31. NEISSER UND WECHSBERG. *Ztschr. f. Hyg.*, 1901, 36, p. 299.
32. CALMETTE. *Compt. rend. Acad. d. Sc.*, 1902, 134, p. 1446.
33. SACHS. *Wien. klin. Wchnschr.*, 1905, 18, p. 901.
34. PASCUCCI. *Hofmeisters Beitr. z. chem. Physiol.*, 1905, 7, p. 457.
35. RANSOM. *Deutsche med. Wchnschr.*, 1901, No. 113, p. 194.

ON AUXILYSINS.*†

A PRELIMINARY COMMUNICATION.

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MUCH light has been thrown on the nature of the immune substances in serum and body fluids by the discovery of simple chemical compounds capable of inhibiting or neutralizing these substances.¹ Although the study of these elementary antihemolysins, antibacteriolysins, and the like, may eventually lead to practical results, their direct application in medicine seems remote. If, however, substances of the opposite nature can be found, substances capable of increasing or augmenting the action of such serum and body fluids, their immediate application in surgery, obstetrics, and internal medicine may be predicted.

During the course of experiments undertaken for an entirely different purpose, a substance of this nature has been discovered. This substance is capable of producing a marked increase in the hemolytic and hemagglutinating action of hemolytic serum.

The action of this auxihemolysin‡ and auxihemagglutinin (*αὐξέιν*, to increase) is still under investigation, and will be reported in full later. A few facts, however, sufficient to call attention to the existence of auxilysins, will be given here, in the hope of stimulating search for members of this group of therapeutic value. An auxibacteriolysin or an auxopsonin would promise much in practical medicine.

If normal goat serum is heated to 56° C. for 30 minutes, it loses completely its power to reactivate a hemolytic goat serum that has been rendered inactive by heat. The complement originally present in the normal serum is completely destroyed by such heating. The normal serum, however, is still capable of exerting a considerable influence on lytic action, as will be shown in detail in a later paper.

* Received for publication February 4, 1906.

† Work aided by a grant from the Rockefeller Institute for Medical Research.

¹ *Jour. Infect. Dis.*, 1904, 1, p. 112.

‡ For suggestion as to the use of the prefix *auxi-*, I am indebted to Professor H. A. Hoffman, Department of Greek, Indiana University.

If, however, the heating is continued for three or four hours, the serum undergoes still further changes and acquires the new property of being able enormously to increase the action of the hemolytic serum to which it is added. On prolonging the heating beyond a certain maximum, this auxilytic power is decreased.

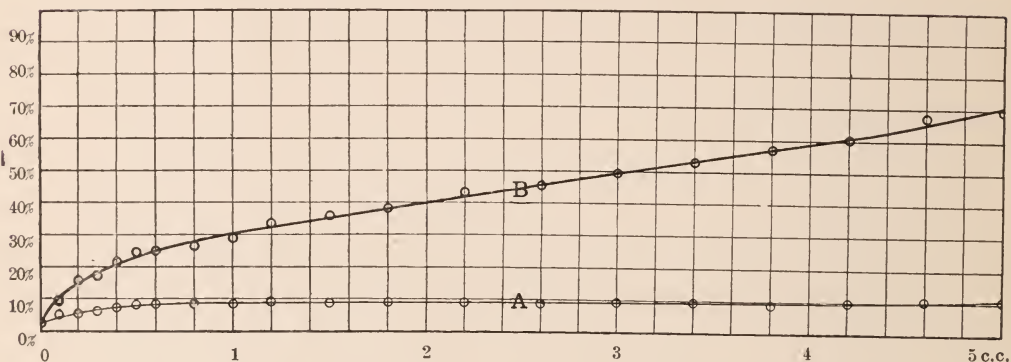


FIG. 1.—Auxilytic Curve. A=Curve showing changes in hemolytic power as increasing amounts of normal serum, heated to 56° C. for 45 minutes are added to a constant amount of hemolytic serum. B=Curve showing the auxilytic action of the same serum, when heated to 56° C. for 4 hours. The hemolytic serum of this experiment, which in itself is capable of giving but 3 per cent hemolysis, has its power increased to 70 per cent by the auxylisin. The auxylisin in itself produces no hemolysis, is incapable of reactivating heated hemolytic serum, and gives no hemolytic power to normal serum.

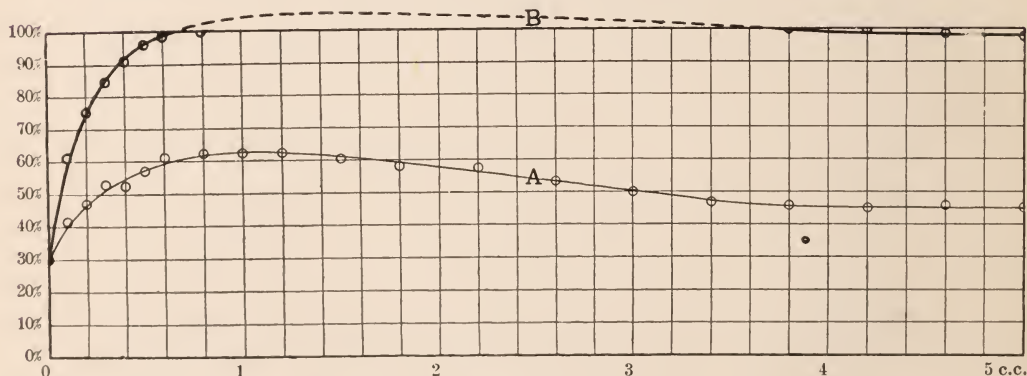


FIG. 2.—Auxilytic Curve. A=Curve as in Fig. 1, obtained with normal serum, heated to 56° C. for 45 minutes. B=Curve showing the auxilytic action of the same serum, when heated to 56° C. for $3\frac{1}{2}$ hours.

The action of this auxilytic substance is shown graphically in Fig. 1.¹ This represents the increase in hemolytic power caused by the

¹ For technique see *Jour. Infect. Dis.*, 1905, 2, p. 460.

addition of increasing amounts of the auxilysin to a constant amount of hemolytic serum. Similar curves are shown in Figs. 2 and 3. In Fig. 3, in place of the constant amount of hemolytic serum, a constant amount of an artificial hemolytic amboceptor-complement mixture was used.

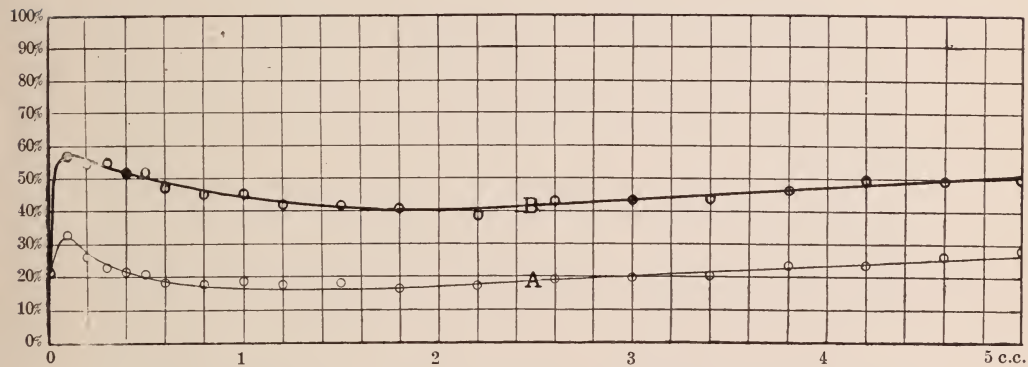


FIG. 3.—Auxilytic Curve. *A*—Curve as in Figs. 1 and 2, obtained with normal serum, heated to 56° C. for 45 minutes. *B*—Curve showing the auxilytic action of same serum, when heated to 56° C. for 4 hours. This experiment differs from those of Figs. 1 and 2, in that, in place of a constant amount of hemolytic serum, a constant amount of artificial hemolytic amboceptor-complement mixture was used.

The immune serum used in this work, possesses not only hemolytic, but hemagglutinating properties as well. This hemagglutinating power is preserved after the destruction of the hemolytic properties by heat. Although accurate measurements have not yet been made, rough determinations show that the auxilytic serum possesses marked auxagglutinating properties as well; the heated hemolytic serum to which it is added being capable of agglutinating corpuscles much more rapidly and completely than normally. Accurate measurements of this auxagglutinin will be made later.

SUMMARY.

Normal goat serum, heated to 56° C. for three or four hours, acquires marked auxilytic and auxagglutinating properties, when tested with goat serum immunized against sheep corpuscles.

A NEW SPECIES OF TRYPANOSOME OCCURRING IN THE MOUSE *MUS MUSCULUS**

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WHILE engaged in a study of trypanosomes in rats in Panama, the writer noticed that not only were these animals infected, but that a certain species of mouse, *Mus musculus*, also harbored a trypanosome.

Up to this time mice have not usually been regarded as being naturally infected with trypanosomes, although Dutton and Todd¹ described a parasite in certain mice in Senegambia which is more or less closely related to this class of parasites. Their organism was described in the fresh-state only, and according to them had no undulating membrane. With the exception of this organism the writer knows of no trypanosome characteristic for mice as *Tr. Lewisi* is characteristic for rats. This is in a certain way remarkable, placing the mice, as it does, in a rather unique relation to other rodents, most of which harbor organisms which are more or less characteristic for each species or family.

The mouse trypanosome is as a rule slender, more so than *Tr. Lewisi*, having a rather pointed end posteriorly, more attenuated anteriorly. The posterior end is somewhat contractile, so that this portion of the parasite varies slightly in its outline, with a corresponding change in length. This, however, is noticeable only in rare instances particularly when the parasite is beginning to degenerate. The difference in length is very slight, and is of no diagnostic importance.

The organism is about 3 μ in diameter, varying as a rule between 2 and 3.5; the length is quite as variable as that of the rat trypanosome, from 10 to 16 μ . Not infrequently one sees smaller or larger organisms than this, but the forms commonly met with lie within the above limits.

The parasite is elongated, vermiform, with a rather conspicuous,

* Received for publication March 6, 1906.

¹ *Thompson Yates Laboratory Reports*, 1903, 5, pp. 56, 57.

highly refractile granule near the posterior end, lying apparently near the centrosome, with a less highly refractile body in the center of the organism. The latter is the nucleus, and occupies the greater part of the diameter exclusive of the undulating membrane.

The motility is very great, resembling that of *Tr. Lewisi*; the organism darts across the field in fresh preparations with great speed, and only after two or three hours does it become slowed sufficiently to permit one to observe the morphology of the trypanosome. Coincidentally with the slowing of the motility, the character of the motion changes, and becomes serpentine; the organism assumes a rapidly changing succession of forms, resembling greatly the letter S.

Along the free border of the undulating membrane, which is well developed, beginning at the centrosome (visible in well-stained preparations) and terminating 3 or 4 μ anteriorly beyond the parasite, is the flagellum, which is in living specimens always in active motion.

The protoplasm, except for the refractile spot and the nucleus, mentioned above, is homogeneous, even under the highest powers, with an occasional granule. As the parasite becomes exposed to unfavorable conditions, the character changes, and one observes a granulation which increases both in size of the particles and the amount of protoplasm so changed, until finally the whole organism becomes a mass of granules.

Division occurs by a thickening of the parasite, splitting of the centrosome, and the gradual formation of a new organism smaller than the parent, adherent posteriorly for a time precisely as in *Tr. Lewisi*. Transverse division has never been observed.

In well-stained preparations the body of the organism is pale blue, the undulating membrane blue, the flagellum red, centrosome dark red, with the nucleus slightly darker in color than the flagellum. The centrosome is, as a rule, slightly fusiform, with the axis at right angles with the long axis of the parasite. The nucleus is in young forms round, becoming more elongated previous to division. Stained preparations as well as fresh organisms are very similar to the rat trypanosome.

In all the cases observed so far there has not been one that exhibited the phenomenon of agglutination. Various sera, both of

mice infected with this organism, rats infected with *Tr. Lewisi*, and non-infected rats and mice, failed to produce clumping of the parasites. In fact, even with the very large number of rat trypanosomes brought to the laboratory there has not been an instance of agglutination.

This organism is not uncommon in the mice of Panama. It has been observed in six out of 66 live mice, and 42 out of 512 dead mice. There is a tendency toward seasonal distribution, being more common in November and December, the last two months of the rainy season, than in the dry season. The same tendency has been observed here with respect to *Tr. Lewisi*. In fact there is an extremely strong resemblance morphologically between the mouse and rat trypanosome—so great that the only safe differentiation is in the source of the organisms.

The criterion upon which the specificity of this parasite is based is the fact that it never occurs in rats; or, more correctly, we have never been able to infect rats with this organism, although the rat trypanosome has been transmitted from rat to rat without difficulty. The material injected into the rats has been shown microscopically to contain actively motile organisms, and from the last half of a hypodermic syringe of material, after injecting one rat, a mouse was successfully infected. The rat proved refractory.

Not only are rats refractory to this organism, but mice themselves are very difficult to infect. After repeated trials two mice, previously shown to be free from parasites, were successfully inoculated. In three days after the first appearance of the trypanosomes, although the infection was fairly heavy, the organisms disappeared, and did not reappear for a month, at the end of which time the animal was killed. The blood at this time, as well as the internal organs, was negative, and the blood was non-infective for mice.

The behavior of mice is unique; the animals do not seem to suffer from the trypanosomes, and, aside from a slight depression and slower response to external stimuli, which is scarcely perceptible, the animals appear perfectly normal and healthy. In no case have infected mice died while under our observation, and animals which have been kept a month after the disappearance

of the parasites (which occurs quite as rapidly in naturally infected as laboratory infected animals) have remained perfectly normal.

The specificity of this organism, then, is based upon:

1. Its occurrence in the mouse.
2. Its non-infectiveness for rats, both wild and white.
3. Its unique behavior in infected mice, i. e., rapid disappearance.
4. Its lack of pathogenicity.

The writer proposes to call the organism *Trypanosoma musculi*.

In conclusion the writer wishes to express his thanks to Col. W. C. Gorgas, chief sanitary officer, the officers of the Panama Board of Health, and the director of the Bureau of Animal Industry; the former for material assistance in collecting material, the latter for identifying the species of mouse.

The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 3

May 18, 1906

No. 3

TWO CASES OF RELAPSING FEVER; WITH NOTES ON THE OCCURRENCE OF THIS DISEASE THROUGHOUT THE WORLD AT THE PRESENT DAY.*

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INTRODUCTION.

THE first of these two cases, exceptional not only in its occurrence but in some of its clinical features also, shows that other cases occur from time to time which are mistaken because of the mildness of the symptoms or insufficient care in examining the blood.

There are no other cases on record in this country up to the present time in which *Sp. Obermeieri* has been found in the blood.

The origin of the first case has not been directly traced, but the existence of cases in tropical America, hitherto unsuspected, seems to indicate its source and makes its appearance here less surprising.

The second case was the result of accidental inoculation.

CLINICAL HISTORY.

CASE I.—C. N., 39 years of age, an Englishman and by occupation a ship's steward, walked into Bellevue Hospital at 9 A. M. on August 15, 1905, and was admitted to the Third Medical Division.

Previous history.—He arrived in New York on August 11, 1905, on a steamer of the Galveston Line plying between New York, Galveston, Tex., and Key West, Fla. He had served three trips on this steamer as steward, being five days at sea and three days in Galveston each trip. He knew of no cases of illness on board nor in Galveston nor in his boarding house in New York. Prior to this he had spent six

* Received for publication March 5, 1906.

months in Santo Domingo and Porto Rico, had made several voyages to South America, and the West Indies during the last five years, and had not been sick during this time. Most of his sea life previously had been spent between England, China, and the East Indies. Twelve years ago (1893) while in the coasting trade out of Bombay and Calcutta had malaria (?) fever. The attack lasted six weeks. He never had had typhoid fever nor syphilis.

Present illness.—On August 13, 36 hours previous to admission, he had a distinct chill lasting half an hour, and following the chill some sweating, continuing probably an hour and a half. He had severe frontal headache and did not sleep well that night. On the next day, though he had no chill, he suffered from chilliness, headache, and thirst. That night he felt weak and could not sleep and concluded to go to the hospital the next day. From the 12th he had had three thin stools each day; no vomiting; no epistaxis; no cough.

On admission his rectal temperature was 102.2° F. and pulse 96, regular and of good force, the chief complaints being severe frontal headache, general aching pains, and some stiffness of the legs. On his face, neck, and upper part of trunk there was an erythematous blush; a few very small pink macules which disappeared on pressure and returned were seen on the trunk. Mucous membranes appeared normal; lymph nodes in neck and groins were just palpable. Tongue was moist, broad, slightly tremulous, with slight brown coating. The spleen was palpable and somewhat tender on pressure. There was little tympanites and some slight tenderness over the descending colon. A few sibilant râles were heard over both lungs. There were no other objective symptoms nor abnormal signs. He was a spare man, fairly well developed and nourished, and weighed 130 to 135 lbs. He was placed on a milk diet at first. Medication throughout his run of fever was placebo.

During the afternoon of the day of admission patient had a slight chill followed by a fall of temperature to 98.5° at 1 A. M. of the 16th. The leucocyte count was 7,800, Widal reaction negative, 1 to 10. (The differential blood counts are given in the table below.)

August 16. Temperature at 3 P. M., 101.6°, pulse 92, respirations 24. No malaria parasites seen on careful search. Urine: 30 oz., sp. gr. 1014, faint trace of albumin, few small hyaline casts; no diazo-reaction. The temperature declined steadily after 3 P. M. to 96.4° at 5 A. M. on 17th, the pulse receding to 52. Patient fairly comfortable with hot bottles and blankets. Has had two or three loose stools during the past two days.

On August 17, 18, and 19 patient was comfortable, no headache, no abdominal tenderness or distension; stools formed. Spots have disappeared. Spleen is palpable. The pulse reached 48, temperature subnormal.

August 20 (seventh day). At 3 P. M. temperature 98.5° F., pulse 56. The second paroxysm now began, the fever rising steadily until by noon of 21st it reached its highest point, 104.4° F., pulse 96, respirations 24. Bowels constipated, headache, shiverings, and general discomfort. Total urine 44 oz.

Examinations for malaria parasites were unsuccessful. Temperature declined sharply (97° F. by 9 P. M.), with some sweating. Pulse 52, respirations 18.

August 24, 9 P. M. (three days after last paroxysm). The temperature which had been subnormal for three days rose to 100.2° F., pulse 88, declined quickly to 97.2° F., pulse 68, and by midnight of 25th had reached 96.5° F., pulse 56. Meanwhile the spleen remained enlarged, bowels constipated. There was no eruption. Three

days later, 27th, a similar rise to 99.6° with acceleration of pulse was noted, and disturbed sleep.

With these two exceptions the temperature range was subnormal almost continuously (a few times reaching 98.5° F.) for nine days until 6 P. M. of August 30. Patient was out of bed and on regular diet. During this interval the patient's temperature was taken every three hours and daily searches were earnestly made for the malaria hemamœba by every member of the staff, all without success. It was this persistent scrutiny, however, that was finally rewarded by the discovery by the house physician, Dr. Heitlinger, of spirochetes in a stained smear made just after noon of August 31—temperature 102.4°. He found six in one smear ($\frac{1}{2}$ immers., oc. II).

This, the third distinct paroxysm, began at about 6 A. M. August 30, and continued for 36 hours. At the beginning he asked for an extra blanket, but he had no distinct chill at any time during the pyrexia. He had severe headache and said he felt "mean all over;" he says he can tell when the attack is coming on because he dreams the night before and is wakeful.

The spleen is tender and more easily palpated. During the decline of temperature there was a return of the looseness of bowels; five stools. Blood taken during night of 31st and forenoon of September 1 contained about six spirochetes to each smear. On September 2, 24 hours after the crisis, one spirochete was found after careful scrutiny of four smears. Highest temperature of this paroxysm was 103° F. Crisis occurred in early hours of September 1, and by 9 A. M. the temperature was 98.5°.

The pulse ran higher during this paroxysm than in first two (112 to 118). Promptly after the crisis the patient felt well, diarrhea ceased, splenic tenderness subsided but the spleen remained palpable.

On and after September 2, the patient was up and about every day during the rest of his stay in hospital (till November 9) except at the time of the fourth and last period of pyrexia (September 9 to September 11). A constant watch was kept every three hours on his temperature until October 6. Weight September 2, 130 lbs.

There is nothing notable of this last intermission except that the spleen remained palpable and patient's weight increased to 134 lbs. His appetite improved; he felt well. He had been placed on a tonic of Fe et Potas. Tart. and Port wine.

September 9. During night of September 9 and 10 patient was restless and could not sleep; had severe headache; felt cold but had no chill; temperature at 5 A. M., 97.6° F., pulse 80, respirations 28. Fever began and by midnight of 10th reached 103° F., pulse from 112 to 120. Face flushed, skin hot and dry, erythematous blush appeared as in the first paroxysm. Spleen enlarged and tender; bowels loose. A remission of 2.5° F. occurred during night with some lessening of pulse rate, but at 9 A. M. with a temperature of 100.4° F., pulse was still 108.

The temperature then rose steadily during the afternoon, reaching the highest point of the fever at 7 P. M., 104.5° F., the patient meanwhile being greatly distressed with frontal headache and muscular pains, and had a pretty severe chill. Pulse 124. Crisis was suddenly ushered in at 7 P. M. by sweating and fall in temperature (7.5° in 10 hours) and coincidentally marked amelioration of all the symptoms. Increased looseness of bowels was noted. Weight 131 lbs., a loss of three pounds during the paroxysm. September 13, complained of considerable pain in right shoulder. No objective signs.

SPIROCHETES.

Sept. 10	2 P. M.	Temp.	101°	1	in 13 smears
	10	"	102.8	3	" 2 "
	12	"	103	7	" 2 "
Sept. 12	11 A. M.	"	98	None	" 4 "
Sept. 15	11	"	98.8	"	" 4 "

From this time on convalescence was uneventful. The temperature ranged between 97° F. in the early morning and 98.5° in the evening until the 25th, after that it ran about normal until patient's discharge on November 9, except that several times it rose to 99.6 °F. as on the 19th and 20th (nine days after last paroxysm) and on the 28th, when patient reported as being restless and uneasy. Nothing untoward developed though the spleen could be felt. By October 1, he had pretty well recovered from his anemia, the differential count was nearly normal. No spirochetes could be found. The spleen could not be felt. On discharge patient weighed 152 lbs.*

Each attack of fever that was observed was characterized by prodromic wakefulness and restlessness; chilliness, rapid, though not abrupt, rise of temperature beginning in the forenoon; decided increase of pulse-rate compared with the interval; severe headache moderate enlargement of spleen; high percentage of large lymphocytes (and in the first and fourth paroxysms by an erythematous rash) and ended in a crisis, the rapid fall in temperature to subnormal occurring during night hours with some diarrhea, sweating, and marked fall in pulse-rate. No jaundice was observed nor gastric symptom at any time. The features of the intervals were subnormal temperature, slow pulse, and rapid return to a feeling of well-being. The patient was not very sick at any time.

Spirochetes.—The organisms found were to all appearances identical with *Sp. Obermeieri*. The accompanying photographs (1500 diam.) furnish the best description, all three of which were taken from the same microscopic slide. The organisms were not very numerous. They were first seen in the stained specimen (Wright's stain). They varied in form and size; they appeared in increasing numbers during the access of fever and disappeared rapidly after the crisis. Only once were any seen during the interval, as is noted above. This was about 36 hours after the crisis. Three days later the unsuccessful inoculation was made.

No cases developed in the ward or hospital as a result of the

*I take this opportunity of thanking Drs. Heidinger, Rimer, and Satchwell, of the housestaff for their unremitting care and attention given in this case.

presence of this case which was treated in general ward, no special means being taken to prevent infection.

CASE I.
URINE EXAMINATIONS.

Stage of Disease	AUGUST					
	16	17	19	21	29	31
	1st Parox'm	Day after Crisis	1 Day before Parox'm	2d Parox'm	1 Day before Parox'm	3d Parox'm
C.c. in 24 hours.....	887	1,124	1,124	1,301	887	1,124
Sp. Gr.....	1.014				1.010	
Urea 24 hrs., grammes					7.77	
Reaction.....	Ac.				Ac.	
Albumin.....	Faint Tr.				None	
Sugar	None				None	
Diazo.....	None				None	
Microscope.....	A few small hyaline casts				A few small hyaline casts	
Diet.....	Milk	Milk	Milk	Milk	Unrestricted Diet	

Stage of Disease	SEPTEMBER					OCTOBER
	1	7	9	11	12	2
	Day after Crisis	3 Days before Parox'm	1 Day before Parox'm	4th Parox'm	Day after Crisis	21 Days after Crisis
C.c. in 24 hrs.....	1,360	2,070	2,830	1,715	2,120	1,479
Sp. Gr.....		1.015	1.014		1.012	1.010
Urea 24 hrs., grammes		22.68	27.09		20.09	9.72
Reaction.....		Ac.	Ac.		Alk.	Neut.
Albumin.....		None	None		None	None
Sugar		None	None		None	None
Diazo.....						
Microscope.....		Neg.	Neg.		Cryst. and Amorph. Phosph.	Neg.
Diet.....		Unrestricted Diet				

On September 5, four days after a paroxysm and three days after the last spirochete was found, a portion of 7 c.c. of blood drawn from the arm of this patient was injected directly subcutaneously into a monkey (*Macacus rhesus*). There was no reaction. On September 10, during the access of the last paroxysm, 5 c.c. was withdrawn from the patient and 3 c.c. injected subcutaneously.* It

* The further results of this and other animal inoculations are reported by Dr. Norris and his assistants, Drs. Pappenheimer and Flournoy, in the article that follows in this number of the *Journal*.

was during the manipulations incident to these experiments that the second case developed. The clinical account of this I present in the words of the patient himself.

CASE II.—The patient was one of the writers of the article that follows. Infection probably took place through being bitten by one of the monkeys, during the latter's paroxysm. After the fracas incidental to having their temperature taken, the monkeys frequently bled from the gums, and it is reasonable to suppose that spirochete blood was inoculated with the bite in this way. This may have occurred on one of several occasions, and therefore the exact period of incubation cannot be made out with certainty.

Family and previous history have no bearing upon the case, and may be omitted.

On the afternoon of October 8, the patient was suddenly taken ill with chilly sensations, severe frontal headache, shooting pains in the extremities, and moderate general prostration. This increased during the night, and the following afternoon he took to this bed. Appetite was completely lost from the beginning. There was no nausea or vomiting, and no icterus; bowels constipated.

The temperature began to rise about 12 hours after the onset of the subjective symptoms, reaching 103° on the afternoon of the second day. It then fell to 100° and remained comparatively low until the fourth day, when it reached 105° ; this was followed after a few hours by a critical defervescence; marked by a drop to normal within 12 hours. The first paroxysm, therefore, lasted four days. Following the crisis there was an apyrexial period lasting for seven days, during which the temperature was persistently subnormal, ranging from 95° to 98° . During the interval there was an absence of all subjective symptoms. The patient was out of bed on the second day, regained strength and appetite rapidly, and was completely recovered before the onset of his first relapse.

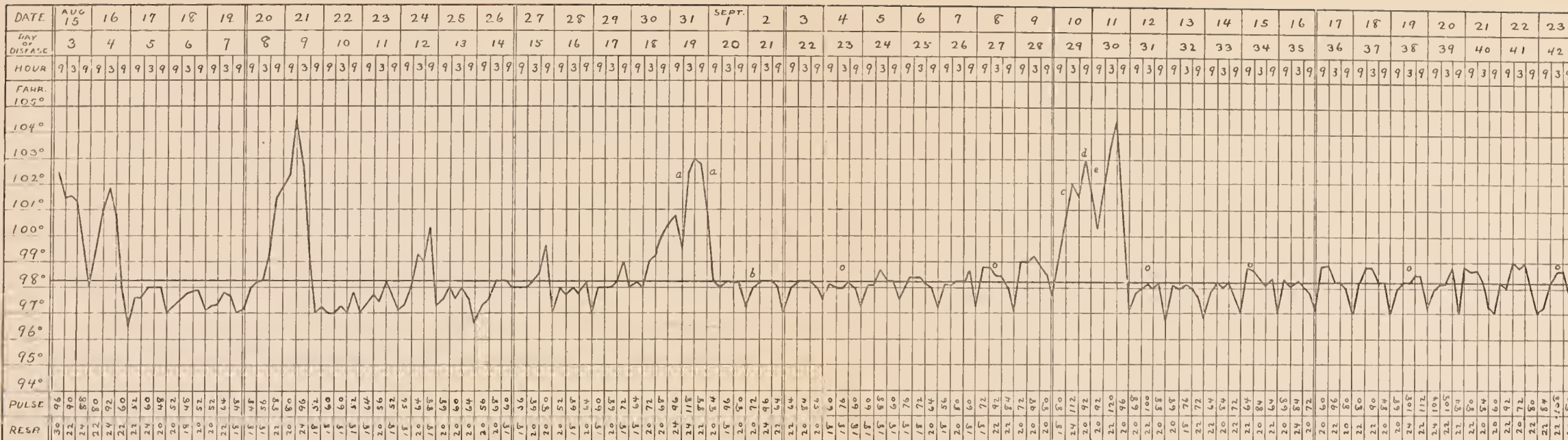
On October 18, seven days after the first crisis, there was a recurrence of the subjective symptoms marking the onset of the first attack. After a prodromal period of about 12 hours, the temperature again rose, reaching 102° on the afternoon of October 19. During the ensuing four days, there was remittent pyrexia. On the morning of October 21, the temperature fell to normal, to rise again to 101.8° during the day. The following morning it fell to 95° , then rapidly rose, and at 8 p. m. reached its highest point, 105.4° , to fall by crisis to subnormal during the night. These remissions in the temperature were attended by a transient release from all feelings of illness and discomfort.

The second paroxysm, therefore, lasted for four days. There succeeded a second apyretic interval of eight days.

At the end of this period there was a recurrence of the usual symptoms, accompanied by a febrile paroxysm lasting two days. On the morning of the second day, there was a remission to normal, followed by an abrupt rise to 105° , and an equally abrupt crisis during the following night.

The third paroxysm, therefore, lasted for but two days. The temperature remained subnormal for several days, then gradually rose to normal. Convalescence after the third attack was rapid, and progressive. Only for a few hours, on the 12th day after the final crisis, was there a recurrence of the sense of nervousness and malaise

RELAPSING FEVER, CASE I, C. N., BELLEVUE HOSPITAL, August 15, 1905.



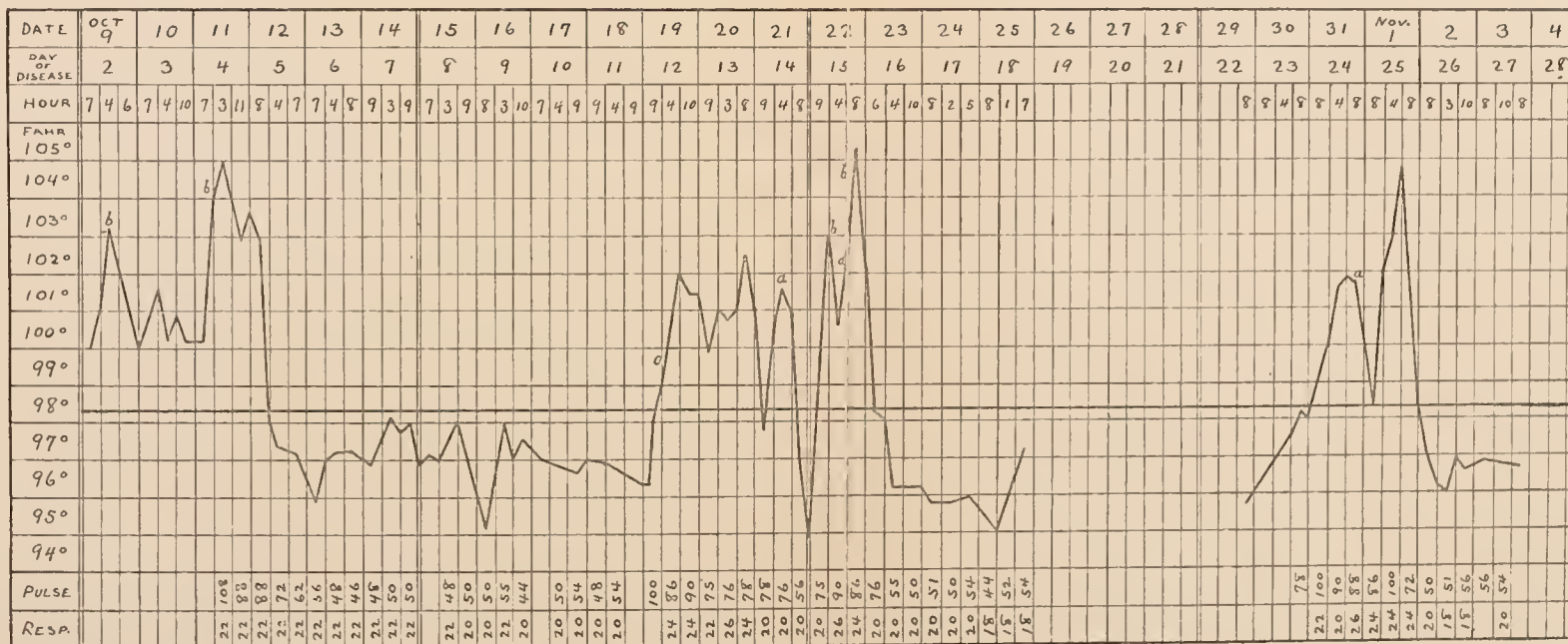
No malaria organisms in daily search.

a, 6 spirochetes in 1 smear.
b, 1 " " 4 smears.

c, 1 spirochetes in 13 smears.
d, 3 " " 2 "

c, 7 spirochetes in 2 smears.
d, No " " 4 "

RELAPSING FEVER, CASE II, October 8, 1905.



a, Spirochetes present, not numerous.

b, Spirochetes present, fairly numerous.

No records taken.

o, Spirochetes absent.

marking the onset of the previous attacks. There was no febrile reaction, however, and this may have been an incidental condition unrelated to the illness.

The course of the disease as outlined above is fairly typical of the relapsing fever caused by the *Sp. Obermeieri*. The duration of the febrile paroxysms is somewhat shorter than the average; yet great variations in the length of the pyrexial and apyrexial periods are known to occur.

Certain individual symptoms may be commented upon. The pulse-rate throughout the febrile period remained relatively slow, as compared with the temperature. It rarely exceeded 100; ranged usually about 80, and on one occasion with a temperature of 105.4°, was only 76. A marked bradycardia accompanied the subnormal temperature between the paroxysms, the pulse-rate ranging from 40 to 50.

Chilly sensations were frequent throughout the greater portion of the fever; during the first relapse, with a sudden rise of temperature, there was a severe, shaking chill which lasted about six hours. This was the only real chill that occurred.

Darting pains in the limbs, and especially in the inguinal regions, were present with each attack, and at times were quite distressing. A dull pain in the back, and in the splenic region was also felt at times.

The sudden falls of temperature were attended by very profuse sweating, usually coming on during the night. These drenching sweats also persisted for several nights after each crisis.

The spleen, examined daily, was palpable only once (October 20, first relapse), and did not appear to be much enlarged. Slight edema about the eyelids was seen in the early mornings, but disappeared after a few hours.

The urine showed a trace of albumin and a few granular and hyaline casts. The excretion of urea was increased. The diazo-reaction was negative.

The blood naturally showed the most interesting changes. Spirochetes were present during each febrile period, disappearing after the crisis. They were never numerous, however, and during the last paroxysm were found but once out of four examinations, and in very small numbers; this, too, in spite of the high temperature and severe constitutional symptoms.

The leucocytes counted on October 9, the second day of the illness, were 14,400. Unfortunately no subsequent counts were made.

A marked and striking relative, and probably also an absolute, increase in the number of large mononuclear cells, or megacaryocytes, occurred with each attack. This is well shown by the following differential counts, made at various times during the illness. Transitional forms and degenerated polymorphonuclears were also in excess during the febrile period.

Aside from this the blood showed little of interest. Polychromatophilia of any degree or granular degeneration was not present.

Convalescence after the second relapse was exceedingly rapid, and save for slight loss of weight and weakness after severe exertion, the patient was in normal health within a week after his final crisis.

Treatment was entirely symptomatic, and apparently in no way influenced the course of the illness.

RELAPSING FEVER.

BLOOD RECORDS,*

CASE I. 300 WHITE CELLS COUNTED.

Day of Disease.	3	19	21	23	27	30	38	50
Stage of Disease.	1st Parox'm 3d Day	3d Parox'm 1st Day	3d Inter. 2d Day	3d Inter. 4th Day	3d Inter. 8th Day	4th Parox'm 2d Day	Last Apyrex'l Period	Last Apyrex'l Period
Time and Tem- perature	1-3 P. M. 101.5°	1-3 P. M. 102.5°	1-3 P. M. 98.2°	1-3 P. M. 98°	1-3 P. M. 98.8°	1-3 P. M. 103°	1-3 P. M. 98.4°	1-3 P. M. 98.6°
No. of reds				4,100,000 [†]		3,400,000	3,600,000	4,480,000
No. of whites	7,800			5,600		5,700	7,600	7,600
Ratio				1:732		1:596	1:473	1:589
Hb % (Tal'quist)				80		70	70	90+
Polymorph	73%	50%	54.2%	55.6%	59.6%	62.1%		64%
Small lymph	25	17.3	10.8	7.2	2.3	5.5		
Large lymph		27.5	30.6	31	27.9	24.5		33
Mononuc. and transition								
Eosinophile	1	5	4.2	5	5.7	5.5		1.5
Mast cells	0.5	0.1	0.1	1	3.5	1		0.5
	0.5	0.1	0.1	0.1	1	1		1

CASE II. 100 WHITE CELLS COUNTED.

Day of Disease..	2	6	12	14	15	24	25	25
Stage of Disease.	1st Parox'm 2d Day	1st Inter. 1st Day	2d Parox'm 1st Day	2d Parox'm 3d Day	2d Parox'm 4th Day	3d Parox'm 1st Day	3d Parox'm 2d Day	3d Parox'm 2d Day
Time and Tem- perature	P. M. 103°	P. M. 97°	12 M. 99°	P. M. 101°	P. M. 103°	11 P. M. 100.8°	1 P. M. 101.8°	7 P. M. 104.8°
No. of reds								
No. of whites	14,400							
Ratio								
Hb % (Tal'quist)								
Polymorph	Incre'd	40%	71%	61%	68%	67%	71%	79%
Small lymph		6	6	3	3	3	4	1
Large lymph		9	16	3	1	9	1	2
Mononuc. and transition		43	4	35	32	21	24	17
Eosinophile		2	4	1	1		0.	1
Mast cells								

SUMMARY.

Case I

Duration of paroxysms 3, 1½, 1½, 2 days
 Duration intermissions 3, 9, 9 days
 Highest rectal temperature recorded 105.5° in last relapse
 Lowest rectal temperature recorded 96.4° in first crisis
 Highest pulse-rate recorded 124 in last relapse
 Lowest pulse-rate recorded 48 in first crisis

Case II

Duration of paroxysms 4, 4, 2 days
 Duration intermissions 7, 8 days
 Highest rectal temperature recorded 105.4° in first relapse
 Lowest rectal temperature recorded 94.8° in second crisis
 Highest pulse-rate recorded 110 in second relapse
 Lowest pulse-rate recorded 44 in first and second crises
 Greatest fall in temperature at crisis 7.5° in 10 hours 8.8° in 17 hours
 Greatest fall in pulse-rate at crisis 124-68=56 at last crisis 110-47=63 in last crisis

* All the blood-counts of Case I were made by Dr. Satchwell of the interne staff; those of Case II by the patient himself.

† Marked granular degeneration of the red cells.

On a cursory view of the temperature and other features of Case I, there is nothing suggestive of relapsing fever to anyone not well acquainted with this disease, and even then he might miss the diagnosis, for so good an observer as H. Vandyke Carter, speaking of the first cases he saw in 1885-86, says, "without special attention it is always possible that genuine spirillum fever may be confounded under 'Remittents.'" But when a spirochete is found in the blood which bears a distinct relation to the paroxysms and is identical in its morphology to that described by Obermeier, and when a second case develops by infection from this one which strongly resembles the typical descriptions of relapsing fever, one can hardly avoid the conclusion that the disease in question is really one of true relapsing fever.

The paroxysms in both cases resemble those of relapsing fever in the abrupt onset with chill or chilliness; the frequent recurrence of this symptom just before the crisis at the acme of the attack; the moist skin and sweating during the febrile periods; and in Case II drenching sweats during, and for some time subsequent to, the crisis; the moist tongue—this is mentioned by Murchison as being the rule throughout the attack, and Flint says it is somewhat distinctive of this disease. The severe headache, muscular and leg pains are very characteristic, and arthritic pains without inflammatory or other joint symptoms not infrequently occur. The moderate enlargement of the spleen and the splenic tenderness are other points of resemblance. And lastly, the crisis occurring during the night time far more frequently than during the day time (Lebert, Carter, Strümpell, and others). The intermissions were marked by extraordinarily low temperature and pulse records, especially in Case II, and this for days at a time.

Variations occur in this disease, as Strümpell says, as well as in any other disease, and it is this fact that Case I emphasizes.

SUMMARY OF CERTAIN CLINICAL FEATURES OF RELAPSING FEVER.

The duration of the initial paroxysm.—Murchison says in rare instances it does not exceed three or four days; Flint says, exceptionally, it may be only two days; Carter says it may end at any time after the fourth or fifth day.

The duration of the intermission.—The longest Murchison says was 12 days; it may not exceed two or three days, and it may extend to 12 days or more (Flint); sometimes only four or five days, rarely two weeks or so (Lebert, who believed relapsing fever and bilious typhoid were not identical); from 6 to 12 days and may extend to 15 days (Sandwith).

Duration of relapses.—Many instances could be given of variation in length from a few hours up to, and even longer than, the primary fever.

Number of relapses.—Murchison says five may occur “making in all six paroxysms;” Flint never saw more than one; Lebert says: “I had not formerly believed in a fourth relapse until during the last epidemic several cases presented it undeniably;” and, finally, Strümpell says the more accurately and persistently we take the temperature during convalescence, the oftener do we find slight rises of temperature occurring at intervals late in the history of the case (see chart of Case I and note the remark in history of convalescence of Case II).

Remissions in the temperature.—Often there are oscillations to a considerable degree (Strümpell). In Carter’s 270 cases in 1876–78 pseudo-crises were remarkable in both invasion and relapse, and in his cases in 1885–86 he says the perturbations of temperatures were marked. Other instances might be cited occurring both before and after Obermeier’s discovery.

It should be noted that we can hardly compare the charts of these two cases to those in the textbooks in this respect, as the former represent three or four hour intervals, while the latter generally show only morning and evening variations.

Pulse-rate.—Usually high in the paroxysms, it may vary from 90 to 110, according to Murchison, but he also says there is less correspondence between the pulse and the temperature in the relapse than in the first paroxysm, instancing a temperature of 106° F. with a pulse of 90 (see Case II of this report—temperature 105.4°, pulse 76).

Nausea and vomiting are among the most common symptoms, sufficiently so, as Flint says, to be somewhat distinctive, especially when the disease is contrasted with typhus and typhoid fevers.

In our two cases they did not occur in the first case, but in the second there was some nausea shortly after the crisis. Nor did the great enlargement of the spleen, reported by some, exist in our cases. The spleen as a rule, however, seems to be but moderately enlarged.

Our two cases differed as regards the condition of the bowels; Case I had diarrhea frequently, and in this case sweating was much less prominent than in Case II.

These two cases then show considerable similarity to the clinical history of relapsing fever as presented by the older writers and the variations which do occur are within the limits of variation recognized by them. Care should be taken that a sporadic instance of disease be not made to conform with earlier descriptions, and, on the other hand, the case in question should not be classified as a new discovery unless it fails in some important particular to conform with the previously recognized type, or until some other way is known of identifying the spirochete than by its morphological characteristics.

HISTORICAL AND GEOGRAPHICAL.

That relapsing fever existed was made plain by the British observers of the 18th and the early part of the 19th centuries, especially by Henderson (1843), Wm. Jenner (1849), and their contemporaries, and was so well differentiated from typhus and typhoid fevers that Clymer was able to recognize it in this country in 1844, and Griesinger was able to report that most of the cases of bilious typhoid in Egypt in 1851 were in reality relapsing fever. Later, when in Europe it was seen in epidemic form, it was supposed to be a disease indigenous to the British Isles.

Obermeier's demonstration, which gave the requisite criterion whereby the disease might be known in sporadic cases and variant types, was soon confirmed by Carter in India, who was the first Englishman to find the organism and to produce the disease in animals (monkeys).

So that there are two important periods in the natural history of relapsing fever. One may be said to begin with Henderson of Edinburgh, in 1843, and the other period may be said to begin when Obermeier in 1873 announced the discovery, which he made in 1868, of the organism which bears his name.

British Isles.—The years 1868–71 mark the last appearance of this disease in the United Kingdom, at least in epidemic dissemination. For 15 years before that it had not appeared. Moore, writing in 1891, says he had never seen a case, though he had seen service in two hospitals in Dublin for nearly twenty years, one of them being a large epidemic-fever hospital.

Whereas before Great Britain and Ireland had been an epidemic center, the disease has ceased to prevail there now for nearly forty years.

Russia.—Eastern Europe has had many visitations, especially Russia. It is said to have occurred first in 1833 at Odessa and in 1840 at Moscow (Hirsch, Dehio). A great epidemic raged in St. Petersburg in 1863–65; in Livonia, one of the Baltic provinces, in 1865 and again in 1883–84; Finland, 1865, Siberia, 1866, and Poland, 1868 (Hirsch), and smaller outbreaks over Russia in 1863–68 (Dehio). Clemow, quoting Reitlinger, of St. Petersburg, says it has reached as far north as Archangel, that in 1874 St. Petersburg was the great center, and that yearly reports came from Vologda, a northeastern province which had been formerly free. It existed in St. Petersburg in 1879–82, 1885–86, and 1890–91; Novgorod, 1898; it was rife in Warsaw in 1879 and 1883, in Moscow in 1882, and again in 1894–95 and 1898.

The observations of Gabritschewsky, of Moscow, on the development of immunizing substances (1896) may be mentioned here, Löwenthal's serum-diagnosis (Moscow 1898), and Sawtschenko and Melkich, also on immunity, in the province of Kasan on the upper Volga in 1900.

In 1893–95 the largest number of cases were reported from near the Volga in the southeast, and the smallest from the Baltic (Clemow, Dehio). Odessa has, from first to last, often been an epidemic center. Moschutkovsky of Odessa, 1876–79, produced the disease in healthy man by the inoculation of infective blood. Tictin, writing of the cases occurring in Odessa about 1889 or 1890, says relapsing fever occurs about once in every 15 or 20 years there.

The United States consul-general's office reports the total number of deaths in Russia from relapsing fever in the year 1901, as given by the Medical Department, as 2,466, out of a total of over

700,000 deaths from infectious diseases (including therein 250,000 from diarrhea and dysentery).

In 1905 the disease continued to figure in the mortality records of St. Petersburg, Moscow, and Odessa. The *United States Consular Reports* state for the week ending May 6, 4 deaths and 19 cases in St. Petersburg; for the three weeks ending May 13, 7 deaths and 126 cases in Moscow, and for the fortnight ending May 13, 1 death and 81 cases in Odessa, and yet, as the *U. S. Public Health and Marine Hospital Service Reports* point out, the malady has not been introduced into this country notwithstanding the large immigration.

Germany has been infected principally in the eastern part and probably from Russia (Hirsch). Upper Silesia, according to Murchison, showed cases in 1848, concurrently with typhus. The most important visitations, however, were those that took place from 1868-73 through northeast and south Germany and which gave rise to the brilliant work of Otto Obermeier in Berlin. Appearing first in Silesia in 1867, the disease spread the next year to Berlin, Stettin, Posen, Königsberg, and other cities. Again in 1878-79 other small epidemics occurred in Berlin, Dantzig, Magdeburg, Halle, Dresden, Breslau, and Würzburg, Heidelberg, and Giessen—1879-80. Since then Germany has been free from epidemics.

In 1895, however, there appeared an imported sporadic case, which is especially noteworthy because of the possible bearing it has on the mode of infection in view of the recent work on human tick-fever in Africa. This case was discovered in Hamburg among the emigrants by Dr. de la Camp, who says it is the first case that had occurred in the Emigrant Bureau Hospital.

De la Camp's Case.—The patient, a Persian on his way to the United States, entered hospital July 15, 1895, on the fourth day of his illness. Seventeen days previously he had started from Ooroomeyeh [a city of about 25,000 people, in the north-western part of Persia, 64 miles southwest of Tabreez. The interest of this lies in the fact that the tick *Argas persicus*, which is said to cause disease in man, is principally found in Persia, in the neighborhood of Miana, a place 80 miles southeast of Tabreez]. The journey was made by horses to Tiflis; rail to Batoom, and thence to Hamburg by rail (steamer Batoom to Odessa).

On admission the temperature was 40.4° C., pulse 100; three paroxysms occurred of eight, six, and three days' length, respectively, intermissions of five, and thirteen days' length. Fever in first relapse developed gradually during three days, and during the relapses was quite remittent. Pulse during first and second fever periods was below 100, except twice 120 was noted; during third pyrexial period 120 to 160. Crises

were acute with marked sweats—greatest fall in second, 41° to 34° C., pulse 125 to 64. Each paroxysm was accompanied by headache, and muscle and joint pains, constipation, slight jaundice, enlarged and tender spleen, and a trace of albumin in the urine near the time of crisis, these symptoms disappearing during the intervals when the temperature became subnormal for a day or two. Spirochetes were discovered one hour after the rise of temperature in third paroxysm (12 to cover glass)—not found after crisis. Leucocytosis marked, but less during relapse and absent during intervals. Hb. 55 per cent. The case is reported in full.

Austria.—Relapsing fever, recognized first as bilious typhoid at Cracow in 1847, occurs nowadays especially in Bosnia and Herzegovina. In 1889–90, in the latter province, an epidemic is reported upon by Karlinski at Stolac. This observer made some investigations on the transmission of this disease by bugs. The disease was epidemic in these parts in 1903. Hödlmoser, of Sarajevo, Bosnia, has done some work on serum diagnosis.

In the *Balkans* the disease is not frequently referred to, and according to Clemow in recent years has been absent from Constantinople. The well-known description by Hippocrates, in which he seems to allude to relapsing fever in the island of Thasos, off the coast of Salonica, may be referred to here, and it may be noted that he gives four symptoms occurring at the crisis, namely, epistaxis, enuresis, dysentery, and sweating, that are of good omen.

The disease is said by many to be found occasionally in Greece, the Ionian Islands, and the Ægean Archipelago—Crete also, and Cyprus—in which places it goes principally under the name of bilious typhoid, which Griesinger pointed out and later Engel proved to be often really relapsing fever.

Asia Minor, Palestine, and Syria.—Cases are sometimes discovered in this part of the world. Tictin, at Odessa, in 1889 or 1890, saw a case in a sailor who had just arrived from a voyage which included a stay at Jaffa, where he seems to think the infection originated. Jaffa lies 31 miles northwest of Jerusalem. As the voyage was of some length, however, the infection might have originated on board ship. The latest record is by J. C. Cropper, at Jerusalem, July 15, 1905, who found a spirochete in the blood of a boy 15 years old who had never been out of the country. The boy was reported sick with a “fever of a quartan type.” His temperature was 101° F. and he was not very ill. The organisms were not very

numerous ($\frac{1}{12}$ immers.). G. C. Low, of London School of Tropical Medicine, who examined the slides, reported that the spirochete was probably *Sp. Obermeieri*. Cropper says it is the second case he has seen.

Clemow says relapsing fever was never prevalent in *Transcaucasia*. Isolated cases occurred in 1889-90 in the city of Tiflis. Quoting Pantiukhoff, who wrote in Tiflis in 1898 on the "Statistics of Caucasian Pathology," he says that sporadic cases occurred along the railroad during the years 1894-98.

Of Mesopotamia and Persia, little or nothing is recorded. The disease is not uncommon in Western Siberia in the Government of Tobolsk.

India.—The disease has long been recognized in all parts of India and constantly recurs. The most important epidemic, from a historic point of view, was that which occurred in Bombay and its neighborhood in 1876-78. It was then that Carter made his important announcement confirming the presence of *Sp. Obermeieri*, and stating that "spirillum fever" is identical with relapsing or famine fever or typhus recurrens; that it can be readily communicated to the monkey (*Macacus radiatus*) and is "unequivocally manifested by both marked general symptoms and abundance of the attendant blood parasite," and that "postmortem appearances are equally concordant."

In a letter to the *Lancet* in March 1880, he made the important statement that he had induced spirillum fever in monkeys by inoculating with infected blood taken during the prefebrile incubation period. Seven years after the close of this epidemic Carter saw five undoubted cases of relapsing fever with Obermeier organisms in the blood, that is from September, 1885 to April, 1886, and by January, 1888 was able to report six cases more, proving, as he says, the continued presence in Bombay of this typical disease, and that notwithstanding the general prosperous condition of the local population.

In 1899 relapsing fever was discovered by Waters in the Common Prison at Bombay, the cases having been first reported as plague. The diagnosis was made by blood examination. Cuthbert Christy reports finding in September, 1900, while "at Ahmednagar, perhaps

the healthiest and nicest station in the Bombay Presidency," in cases of an epidemic disease in a near-by village, *Sp. Obermeieri*. In this same village plague had occurred the previous year. The disease, he adds, is endemic in certain quarters of Bombay. He made some observations at this time on infection by bedbugs, of which there are plenty there.

There can be no doubt that spirillum fever, to use Carter's term, is continually present in Bombay. Clemow (1903) says the disease, relapsing fever, was "severely epidemic during the last three years." A. Powell, of the British army, expresses the opinion that there must have been "tens of thousands" of cases in Bombay in 1901-2, and adds that it is strange it is not imported into Europe, the journey to London requiring but 14 days.

Amongst the hill tribes of the Himalayas it has long been known. According to Murchison it is said to have been recognized in 1852 in the valley of Peshawur, and again Hirsch shows that it was probably introduced into the Punjaub by the muleteers returned from service in Abyssinia in 1866. In the Kumaon Hill in 1898, Leon Rogers reports relapsing fever, and finds the *Sp. Obermeieri* in one of a group of cases all having similar symptoms and reported as mild plague by the natives. Also G. Browse reports finding this organism in a case simulating malaria occurring in March, 1904, at Nowshera in the Punjaub, in a well-situated and healthy regimental station. The patient was a cavalryman who had not been away from his station for a year. The case, he says, was exactly like cases diagnosticated as malaria in the Punjaub.

L. A. Walker found the parasite in some cases of a recurrent fever epidemic in the eastern portion of the Mardan district on the right bank of the Indus in 1904.

As to *Farther India and Malay Peninsula* the only mention I have found is that of Dr. James Kirk of Singapore, in a paper read before the Malay Branch of the British Medical Association, Session 1903-4, analyzing 150 cases of local fever. He says: "I have not yet diagnosed relapsing fever here."

East Indies.—A case of relapsing fever is reported in May, 1901, from Sumatra, by J. C. Graham at Deli, in which the spirochete was found. The patient was an immigrant Chinese coolie from

Swatau. He says it is the first case ever reported in Sumatra. The symptoms were high fever, severe headache, pains in the legs and joints, tenderness in both hypochondriac regions, slight icterus. No malaria parasites were present, specimen teemed with "*Sp. Obermeieri*." Crisis on sixth day with fall to 35.6°.

China.—In the north, according to report, various foci existed in times past. In 1864-65 this disease was epidemic concurrently with typhus fever, and Tien Tsin suffered greatly in 1877. It was reported in the province of Shan Tung as very prevalent in 1889 (Neal—no blood verification).

In the south, the most common form of fever at Ting-Chau-fu is relapsing fever, to quote Clemow again.

From the province of Kwang Tung on the southeast coast spirochetic infection in sporadic instances is recently recorded from two places. One of these cases, namely Swatau, was mentioned above. The others are by L. G. Hill, of the Church Missionary Society Hospital, at Pak-hoi on the gulf of Tong King. In July, 1903, in making routine blood examinations of all cases, he discovered in a woman 65 years old "*Sp. Obermeieri*." She had had a fever period of 11 days, an intermission of three days, and fever again for four days according to report, when admitted. A crisis occurred the same night. An interval of nine days followed, then a period of three days' fever, a one-day interval, and a one-day relapse. The organisms were many and active when first found. During the first two days of the first interval that was observed, the organisms continued in diminishing numbers and then disappeared till next paroxysm, when they were again plentiful, but not so active. Though the case was treated in the general ward, no other case developed.

On March 7, 1904, a Chinese coolie, aged 33 years who had been in the employ of a European for several years and who lived in the coolie quarters, was admitted with a temperature of 101°, having been ill several days. Myriads of parasites were found. No malaria organisms—excess of leucocytes. Crisis occurred two days later, the temperature registered 99.8° and no spirochetes were found. March 10, subnormal temperature and no organisms.

Hill says the disease had not been previously met with at Pak-hoi nor at Hong Kong.

In January, 1905, Koch of Hong Kong met with three cases among Chinese emigrants en route from Chin-wan-tao to South Africa. The periods of these cases were as follows:

Case I, two paroxysms of seven and four days with five days' intermission. Case II, fever 12 days, no "remission;" Case III, fever 12 days—sudden death after three days of apyrexia. Numerous "spirilla were found and no malaria." He states he is unable accurately to determine the duration of the first paroxysms. In the fatal case "no spirilla were found in any of the organs or in the blood of the heart. The spleen was enlarged and firm."

As a sequel of these cases the office boy of the hospital fell sick of the disease and went through a typical and severe attack with fever periods of six and ten days, separated by an interval of five days.

This observer says the disease is very rare in Hong Kong.

The Public Health Reports of the United States Marine Hospital Service for June 2, 1905, say that cases occurred in various places in China in the first part of last year.

Japan.—Baelz suggested in 1898 (as quoted by Clemow) that an epidemic disease which he observed in the island of Skikoku in 1881, may have been relapsing fever. Scheube (1903), who was formerly connected with the University of Kioto, does not speak of this disease as occurring in that country.*

Philippines.—I have been unable to obtain verification of the statement of Osler (1905) that these islands have suffered from several severe outbreaks—neither in Hirsch, Davidson, Clemow, Scheube, nor elsewhere.†

Egypt.—After the clinical demonstration by Griesinger, and the microscopical proof given by Engel at Cairo in 1884, we hear little of relapsing fever in Egypt till 1904. Clemow, writing in 1903, says the disease was formerly endemic in Egypt, but that it is absent from reports throughout Africa in general.

A good clinical account of this affection as it is seen in the Delta is given by F. M. Sandwith, of Cairo, in May, 1904. Sandwith found the spirochete in cases reported as typhoid in 1884 in and about Cairo and at Zagazig, an important cotton center 75 miles northwest of Suez. It appears that the disease has been more or

* One or two papers were published at Tokio in 1895 on recurrent fever, printed in Japanese.

† The report of Assistant Surgeon B. L. Wright, U. S. N., in the *Phila. Med. Jour.*, 1901, 7, p. 301, of cases of "Cavite Fever" puts one strongly in mind of relapsing fever. Although no reference is made to this disease in the article it is possible the cases were of that nature.

less continually present ever since. Eight deaths occurred in the provincial prisons in 1902. During the previous 11 years he had altogether 35 cases in his wards in Cairo. Suez, on the highway to the East, is another important center of distribution. Sandwith says the only case he ever saw in a European was at this place in 1892. In July 1905 there is a report of five cases at Cairo imported from Suez, in which the spirochetes were found by L. Phillips. The temperature charts are given. The shortest relapse was three days; the longest interval 10 days.

Abyssinia is noted above in connection with the Punjab.

Tripoli, Tunis, Algeria.—Along the north coast of the African continent the disease has been reported recently by several of the French army surgeons.

A. Billet reported a case in an Algerian soldier, aged 30 years, in September, 1901.

The man came to the hospital at Constantine with the diagnosis of malaria. He had two paroxysms: the first lasted four days; the second one and one-half days; the interval lasted 13 days. A few "Obermeieri" were seen the first day (four or five to field) and they increased to 10 or 15 to field before crisis. There was a prompt disappearance at the crisis. In the relapse a few were found (1 in 10 or 15 fields). A leucocytosis of 26,600 when the temperature was 39° C. on third day: polymorphonuclears, 77 per cent, large mononuclears, 1 per cent, small mononuclears, 22 per cent, eosinophiles, 0.1 per cent, 100 cells counted. The reds numbered 2,511,000. Hb., 85 per cent (Henaque). After the crisis the white cells fell to 19,000 (65 per cent polymorphonuclears) and in second paroxym the count was 17,000 (74 per cent polymorphonuclears). Other features were absence of prodromes, slightly remittent temperature, rapid pulse (120 to 125) and respirations, enlarged spleen, some icterus, dry tongue, delirium, epistaxis preceding crisis, abundant urine and profuse sweat at crisis, followed by subnormal temperature.

Billet says the disease is excessively rare in Algeria. A small epidemic occurred near Constantine in 1866. Friant and Cornet record some cases occurring in Algeria near the frontier of Tunis in December, 1903 and January, 1904.

Lafforgue reports in 1903, from the vicinity of Tunis, 20 cases in which a spirochete was found identical in form and mobility and other characteristics with *Sp. Obermeieri*. He thinks cases probably occur in Tripoli.

Morocco.—It is said by both Davidson and Clemow to have appeared in this country during the periods of famine following plagues of locusts.

Grand Canary.—In the *U. S. Consular Sanitary Report* for the week ending May 6, 1905, 13 cases of relapsing fever were reported in the Grand Canary (Spanish).

Gibraltar.—Here may be noted the case of infection by spirochetes that was reported by Manson in 1904. An English lady, aged 30 years, who, having resided at Gibraltar for three years, returned there after a short visit to England on September 29, 1903. On October 20 she had a rigor followed by a fever (104° F.) which after profuse sweat terminated in four days. A ten-day intermission and a second paroxysm occurred, lasting three days, after five days a third, lasting also three days. Another interval of 10 days, during which time the patient returned to England, and on November 24 a fourth period of fever began. After this paroxysms occurred on December 4, 15, 29, and January 11. Each paroxysm except the last, which was very mild, was ushered in by rigor and terminated with profuse sweating. Spirochetes were found in the paroxysm of December 29, but were not looked for before, except during the subsiding fever of the previous period. They were few in number, and Manson is noncommittal as to whether it was the *Sp. Obermeieri* or not. He thinks there were too many relapses and that the spirochete (of which an illustration is shown) differs in shape from this well-known organism.

The number of paroxysms is greater than in any other case I have found record of.

Tropical Africa.—In the course of the years 1904 and 1905 several clinical and three most important experimental reports were made under the various titles: relapsing fever, spirillum fever, spirillosis, and "tick-fever" from British East Africa, the Congo Free State, German East Africa, and Portuguese West Africa.

Uganda.—A. R. Cook, from the missionary hospital at Mengo, at the head of Lake Victoria Nyanza, published, early in 1904, some notes of cases of relapsing fever.

Case I.—March 21, 1899. A little girl, whose blood teemed with spirilla, was admitted in extremis and died 16 hours later; no postmortem examination. During the next four years isolated instances were seen, and in November, 1903, a somewhat widespread epidemic was noted.

Case II.—November 7, 1903; a man ill three days with vomiting and pains in the chest; no rigor; no diarrhea; collapsed condition; temperature 99.4°, pulse 140, respiration 80; conjunctivæ jaundiced; delirious. Spleen and liver not felt; very

scanty urine, trace of albumin. Fresh specimen of blood showed over 30 spirilla to one field ($\frac{1}{12}$ immers., oc. III); death in four hours. Autopsy three-fourths hours later. Smears from the enlarged spleen showed few spirilla.

Case III.—November 8, 1903. A man three days sick in his second paroxysm. Rigor at the onset. No vomiting nor pains. Temperature 101.6° , rising in one hour to 104.2° ; slight delirium; no jaundice. Blood showed one organism to each field. Crisis on fourth day when spirilla disappeared.

Case IV.—November 15, 1903. Small girl whose blood swarmed with spirilla. Temperature 105° ; no accurate history.

Nabarro, while studying sleeping sickness in Uganda, met with a case of spirillar infection on August 1, 1903.

Hodges and Ross, also in Uganda, discovered the organism in the blood of an Indian who had been absent from India for over a year and who had never had a similar attack before. These organisms were few, sharply pointed at both ends, and were about 40μ long and 4μ broad. The symptoms were vomiting, back-pain, temperature 103.4° , with spleen slightly enlarged and tender. Crisis on the next day; relapse 18 days later when spirilla were again found. A monkey was inoculated. After three and one-half days fever began in the monkey, lasting three days; numerous spirilla. Every case of fever was examined (60) coming from various parts of the Protectorate, and 12 showed spirilla, comparatively few organisms in all. In the one fatal case the spirilla disappeared from the blood 24 hours before death, while the temperature was still high.

Angola.—Portuguese West Africa is a province bounded on the north by the Congo State. From Benguela, a district on the coast of this province, in the early part of last year A. Y. Massey reported a case of spirillosis in a man which he met with in December, 1903. The patient, a Portuguese trader, had a temperature of 103.4° and involuntary passages, and died three days after admission. Fresh specimens of blood showed spirilla innumerable. Next, Wellman, in April, 1905, reported from Angola a case of relapsing fever with spirilla. The patient, a native 32 years old, was admitted March 3, 1904, one day sick; temperature 104.4° , pulse 92, respirations 29. No rigor or vomiting; organisms about a dozen in each field ($\frac{1}{12}$ immers., oc. II). Crisis occurred on the third day. A few spirilla persisted for two days more. After an intermission of seven days a relapse of three and one-half days occurred during which the organisms were fewer in number.

Ile de Réunion and Mauritius.—These islands off Madagascar were infected with relapsing fever in the year 1865, the disease having been introduced direct from India by coolies. It lasted for several years (Hirsch and Stephen Smith).

Uganda (Tick Fever).—Continuing the investigations of Hodges and Ross at Entebbe, Uganda, Philip H. Ross and A. D. Milne, knowing of Marchoux and Salimbeni's demonstration that spirillosis of fowls is conveyed by a tick, thought that perhaps the so-called tick-fever might be due to a spirillum. They were unable to get any reliable description of symptoms, nor good temperature records. Some cases had vomiting and some had relapses; some had splenic tenderness. The most common symptoms were severe headache, pains in back and limbs, and fever ending critically. All had spirilla sparsely scattered and difficult to find. They concluded that certain cases diagnosticated as tick-fever are due to a spirillum. Their observations were published on November 26, 1904. On the same date J. E. Dutton and J. L. Todd cabled from the Congo: "Spirilla cause human tick-fever. Naturally infected *ornithodoros* infect monkey."

Congo Free State.—Dutton and Todd were working independently of Ross and Milne and were on the Upper Congo at Kasongo some distance west of Lake Tanganyika in November, 1904, when they both contracted this disease which later led to the death of Dutton, February 27, 1905. A report of their work was issued last November (1905) and their conclusion is that the tick-fever in the oriental province of the Congo Free State is a relapsing fever, produced by a spirochete, probably identical with *Sp. Obermeieri*, and that this organism can be transmitted by the tick *Ornithodoros moubata*. The report contains records of 14 cases of which two cases were Europeans, Dutton and Todd themselves being the victims. There are also notes of animal experiments, and remarks on the distribution of the human tick in the Congo region, together with notes on the external anatomy of *O. moubata* by Newstead.

Dutton and Todd remark what Livingston said, that the ticks followed the Arabs, and point out that the ticks seem to have come into the Free State by two routes; from the East Coast with the Arabs, and with traders from the Portuguese territory to the south.

A closely allied, if not identical, *ornithodoros* is found in Angola (Newstead).

Cuthbert Christy found these ticks in Uganda in 1902, and says that they are distributed in German East Africa, Angola, and in the Zambesi, and are closely allied to the *O. Savignyi* found in Egypt, Nubia, Abyssinia, and Somaliland.

Milne says that one of the chief caravan roads in German East Africa, south of Victoria Nyanza, had been closed, owing to constant sickness caused by tick-fever. Koch found the ticks widely distributed over the district (1905).

The tick *Argas persicus* is found in Persia and Beluchistan, being especially common, according to Manson, in the northern part of Persia in the neighborhood of Miana. The natives attribute a disease to its bite, which is known as the "disease of Miana." (Compare de la Camp's case noted above.) Manson quotes Schlimmer as saying that on one occasion (1858) he treated 400 soldiers who declared they had been bitten by these ticks at Miana. The symptoms resemble those of "remittent fever—extreme lassitude, disinclination to work, yawning, fever, perspiration, not accompanied by much thirst, increasing and decreasing at stated hours of the day."

United States.—The first introduction of relapsing fever into this country was by Irish immigrants in 1844 at Philadelphia, when Clymer was the first to recognize it, he having 15 cases under his care. Pepper, Parry, and others also saw cases. The disease did not spread far. In 1850-51 Flint differentiated cases from among some Irish immigrants affected with typhus, who arrived at Buffalo where he was then in practice, and he also reported 15 cases, and adds that doubtless others occurred which were mistaken for typhus. It is mentioned by Clymer that, in a report from the Russian government to the English ambassador at St. Petersburg, the disease was said to exist at New Archangel (now Sitka, Alaska) in 1858. The next and last time it appeared here was in epidemic form in New York. The first case appeared in October, 1869. The epidemic lasted through the winter and spring and in rapidly waning numbers into 1871. In Philadelphia numerous cases appeared. The disease did not spread far from these centers, however. A few

cases occurred in Washington, in Maryland, New Jersey, and Connecticut, and one case was imported into Boston, as noted by Shattuck in the American translation of Strümpell's work on "Practice of Medicine."*

In September, 1874, a severe outbreak of epidemic disease was observed at Oroville, California among Chinese laborers. Many of these cases were similar to typhus, judging from the description, but many had the characteristics of relapsing fever; crisis on the sixth day and relapse on the 14th day after a period of complete apyrexia, and are so reported by the observer, Miller.

At Worcester, Mass., in August, 1899 a sporadic case appeared in an Armenian immigrant and is recorded as relapsing fever by Ward. This diagnosis was not confirmed by blood examination, though the spirochete was looked for during the relapse. The case presents all the other characteristic features, however. Malarial organisms were absent.

South America.—Baldou says it appeared in Peru in 1854 and, known as "peste des Cordillères," spread thence slowly to Chili and Bolivia (1856). Hirsch (1881) says nothing is known of its existence in Central and South America.

Cuba.—Relapsing fever is next heard of in this part of the world in 1902, when Biada, of Havana, reports, under the title "recurrent fever," an important case that simulated yellow fever and by a careful examination of the blood was proved to be due to spirochetic infection.

Biada's case.—Spaniard (Espanol) aged 19 years, a sailor for one and one-half months on steamer from Liverpool, to the coast of "Cantabrias" (Spain?), to Cienfuegos, to Havana, and then on a coasting schooner. The onset was abrupt on January 19, 1902, with chill. Temperature 40° C., pulse 116, respirations 30, pains all over, especially in legs; constipation; spleen very large. No malaria parasite was seen in the blood, but perfectly characteristic *Sp. Obermeieri* (three in one field) were discovered by Dr. Kirby Smith. The urine (550 c.c.) contained some albumin on third day. The fourth day much pain in splenic region, diarrhea; fifth day, epistaxis; sixth day black vomit, slight jaundice, crisis—fall from 103° F. to 95.4° from 6 A. M.

* A case of relapsing fever entered the Presbyterian Hospital, New York, last July (1905) in the service of Dr. George A. Tuttle who kindly permits me to mention the fact. The patient, an Armenian woman who had been in this country for a number of years, had entertained, a few days before her sickness began, some relatives newly-arrived from Armenia; they themselves were not sick, however. The patient had two paroxysms of fever, each of five days' duration separated by an interval of like length. The diagnosis was confirmed by the finding of the organism in the blood during the relapse. The case is to be reported in the *Medical and Surgical Reports of the Presbyterian Hospital for 1905-6*.

to 6 P. M.; disappearance of spirilla. On the seventh day felt better; stools formed, urine 325 c.c., less albumin. Eighth and tenth days, urine between 1,400 and 2,200 c.c.; felt fine on 11th day; no spirilla. February 7, 13th day after the crisis, epistaxis returned, urine contained much albumin, and again, six days later, no fever appearing and no organisms, abundant albumin was noted. February 20, slight iritis. He was subsequently discharged without having any relapse of fever, but with some edema of extremities, a little albumin in the urine, and bad color to skin.

The day before the crisis (January 24) a monkey was subcutaneously inoculated with 3 c.c. of patient's blood. Spirochetes were found in the monkey's peripheral circulation 61 hours after inoculation. The animal's temperature ranged between 40° and 39° C. for two days and fell on third day coincidently with the disappearance of spirochetes.

Mexico.—Some cases were noted here in April, 1905. The Surgeon-General's office of the U. S. Public Health and Marine Hospital Service writes in answer to an inquiry, that they were reported from Tuxpam, that deaths occurred from this disease during every month throughout the year, but that the weekly sanitary reports from United States Consul Lesspinasse at Tuxpam do not state the number of cases occurring, nor give other information relative to the deaths that have occurred. Tuxpam is a seaport on the gulf coast between Vera Cruz and Tampico—145 miles north-west of the former place. It has a large trade in cedar, vanilla, fustic, and coffee.

Panama.—The Surgeon-General has very kindly furnished the following statement, dated February 14, 1906: "It is understood that two cases of the disease (relapsing fever) were under treatment in the Colon Hospital, Colon, Panama, during the summer (1905), the diagnosis having been verified by blood examination."

The foregoing account shows that there are cases of periodic fever occurring in many widely separated parts of the world which, in their clinical manifestations and infective character, are strikingly similar to relapsing fever, and, as regards the associated microorganism parallel, if not identical, therewith. In respect of so-called tick-fever, Dutton and Todd, in their report, are as explicit as Carter was. They say (p. 4): "The case reports, charts, and postmortem finding certainly demonstrate the clinical identity of the tick-fever observed by us, with the relapsing fever of the textbooks." Koch says that the relapsing fever seen by him in 1905 in German East Africa is closely similar to the European relapsing fever, the unlikeness being

that the relapses are shorter and the spirochete is, as a rule, longer in the African disease.

MODE OF INFECTION.

Most, but not all, of the older writers believe that relapsing fever is a contagious disease.

Many authorities might be cited and many instances given to show that the disease is not a highly contagious one; that, on the introduction of a case into an uninfected area, the disease does not spread rapidly nor far; that from the existence of a single case in cleanly surroundings, and without special attempts at isolation, the disease is not newly developed.

None of the reasons given, tending to prove that the disease is a contagious one in the sense that typhus fever is said to be, is inconsistent with the view that it is conveyed by blood-sucking insects, such as have been proved to be the means of infection of spirochetic disease, as in so-called tick-fever in man, *Sp. Theileri* in cattle, spirillosis of fowls, etc.

Nuttall says that Flügge, in 1891, was the first to suggest the possibility of vermin serving to spread relapsing fever. Tictin, in 1897, at Odessa experimented with the ordinary bedbugs, and succeeded in causing the disease in monkeys by inoculating them with blood taken from bugs immediately after they had fed, but failed to succeed when an interval of 48 hours was allowed to elapse.

Karlinski found abundant spirochetes in bugs caught in houses in Bosnia, where relapsing-fever cases existed, and none in bugs caught in other houses free of this disease. He says that spirochetes persisted for 20 days in bugs. Dutton and Todd found living spirochetes in the stomach and malpighian tubules of ticks up to five weeks after their feed on a known infected animal, and were able to infect a monkey after one and three-fourths months. One of their conclusions is that the transmission (of the spirochete) is not mechanical, but some developmental process is carried on in the tick.

Schaudinn suggests that certain stages in the life-history of the *Sp. Obermeieri* may be formed in the blood of the patient which in relapsing fever are not recognized as parasites.

Cuthbert Christy at Ahmednegar, India, did not succeed in acquiring the disease by allowing himself to be bitten by bed-bugs caught in the bed of a patient sick with relapsing fever.

In Case I of this report, the disease undoubtedly originated outside of New York. That spirochetic disease existed in the part of the world where this patient has spent much of his time recently has been shown—Havana, 1902, Colon, 1905, and perhaps cases at Tuxpam. No case developed in Bellevue Hospital subsequent to his stay of 89 days in hospital, and absolutely no special means were taken to prevent infection. There are several varieties of ticks to be found in the West Indies, South and Central America (*O. talaje*) and Mexico (*O. turicata*). Sambon has suggested the *O. turicata* in Mexico as a possible factor.

DIAGNOSIS.

There is great approach to unanimity shown by the various observers in the terms employed, namely, relapsing fever, recurrent fever, recurrent typhus or spirillum fever. Furthermore, a study of all these reports and our cases leads one to the conclusion that these cases cannot be differentiated one from another either clinically, or by the morphological features of the spirochete, as far as these are known, or by animal inoculation.

A thorough search of the blood during the pyrexial period, and according to Carter, even during the pseudo-crisis, will reveal the spirochete. These are not very numerous in many of the cases. They increase in number with the progress of the febrile period, and in each succeeding relapse, because of decrease in numbers, more care is required in the search. They begin to disappear just before the crisis, but may be found (in Case I in four large smears and in one of Hill's cases at Pak-hoi and the first two cases of Dutton and Todd) a day or two after the fall. The statement of Sandwith, that the spirilla entirely disappear from 12 to 24 hours before the temperature falls at the crisis, is certainly not true for most of the cases, though Heidenreich, of St. Petersburg, quoted by Fagge, makes a somewhat similar statement. Heidenreich mentions what has been spoken of by many of the recent observers, that there are fluctuations in the number of the organisms during the same

pyrexial period, and differences in mobility, size, and shape may be noted in the same specimen of blood (Carter, Dutton and Todd, and others, and see accompanying microphotographs from the same smear of blood in Case I). Carter and Heidenreich have found the spirochetes during the interval, just before the relapse, and in Cases 8 and 9 of Dutton and Todd the day before the rise of temperature. They were seen in the interval also in one of the native "tick-fever" cases, by Ross and Milne—the patient complaining of severe headache, but without a coincident return of fever. Sometimes when a relapse is about due, the patient complains of some subjective symptom, headache, wakefulness, restlessness, and the temperature is slightly raised (subfebrile), but no spirochetes are found. (Fagge and Cases I and II of this report.) The organisms have been found after death, in the blood and in the spleen (Strümpell, Dutton and Todd's Case IV and Cook's Case II).

The blood, besides the positive proof presented during the febrile period, shows some changes in the leucocytes which apparently vary in greater or less degree, side by side with the progress of the disease.

Many of these cases, in which the number of white cells are recorded, show a moderate leucocytosis (de la Camp, Hill, Billet) particularly distinct in the first paroxysm. Case I of this report, and of the three instances of Dutton and Todd's report in which the note is made, Cases XIII and XIV, do not agree with this, in the other of these three instances, the fatal case, the count was 14,200 and during the crisis 11,800.

It may be said that there is very often recorded, in the first paroxysm, an increase in the number of white cells, particularly the polymorphonuclears, which diminishes early in the interval and is less marked with each recurring paroxysm. This is not invariable, as is shown in the table given above, in which the most marked change is in the relative number of large lymphocytes and mononuclears. No distinct changes in red cells other than those indicative of secondary anemia occur.

A study of these sporadic instances of spirochetic infection suggests that the following symptoms may indicate the probability that this condition is present.

A period of fever of four or five days' length, beginning abruptly and ending abruptly, chilliness or chill at onset, recurring just before the crisis. Sometimes epistaxis at the same periods, very severe headache, backache, and leg pains. Moderate sweating and moist tongue during the fever. Moderate enlargement of spleen, with considerable tenderness on pressure. Pulse increased, perhaps rapid. Recurrence of similar paroxysms at any time from third to eighteenth day, usually between seventh and fourteenth. Relapses short, from one-half to three days as a rule, during which fever may be higher than in first paroxysm. The number of relapses may be from one to seven—perhaps more. Often a pseudo-crisis the day before the true crisis. Crisis accompanied by drenching sweats coincidently with a rapid and very great fall of temperature occurring during the night and extending over 12 or 16 hours.

The interval characterized by temperature and pulse-rate much below the normal standard; this often continuing until the day before the next paroxysm. Sweating persisting often for two days at the beginning of the interval; disappearance of splenic tenderness and quick return to feeling of well-being.

Löwenthal has proposed a serum reaction which (when the opportunity offers) he says will enable the diagnosis to be made during the interval. This reaction lies in the power of the blood serum of a patient who has passed through one paroxysm to agglutinate the spirochetes in the blood of a fever case, a power not possessed by normal blood serum.

Malaria is the disease for which these cases of spirochetic infection are most likely to be mistaken in this part of the world. In a patient presenting symptoms similar to malaria and who has recently arrived from foreign parts or who has been more or less intimately associated with a newly arrived person, a careful search of the blood should be made for this organism in question. It is perhaps allowable to point out that, compared with relapsing fever, malaria presents a more regular incidence of febrile periods; a shorter interval between the paroxysms, and generally a spleen more easily palpated.

The blood in malaria shows an early and a greater loss in the red cells and hemoglobin percentage. As regards the white cells, the importance of the presence of intracorpuseular pigment is to be

kept in mind. The leucocytosis observed by many in relapsing fever is unusual, if not wanting, in uncomplicated cases of malaria.

On the other hand, the normal or diminished white-cell count associated with a very high relative increase in the larger lymphocytes and mononuclear cells seen in our cases, has been seen by many in malaria (see Stephen's and Christopher's note quoted in Mannaberg's article in Nothnagel's *Encyclopædia*).

Mannaberg quotes Mamourski as having seen a case which showed both a malaria parasite and the spirochete of Obermeier in the blood at the same time.

Influenza is suggested by the sweating and the marked subnormal range of temperature characteristic of the intermission. Rabagliati says he knows of no other diseases in which this feature is so apt to be emphasized as relapsing fever and influenza. In the latter disease a relapse, if it occurs, follows the first febrile period after an apyrexial stage of much shorter duration than that that rules in relapsing fever, and the symptoms in such a case do not promptly or entirely disappear on the fall of temperature.

Considering the fact that the vomiting of blood, so common in yellow fever, may occur in spirochetic infection (Biada's Case), and that epistaxis and jaundice occur in the latter condition, one should always look for this organism in the blood of cases showing some of the characteristics of yellow fever. A statement similar in effect may be made of cases that suggest the presence of plague, typhus fever, or sepsis.

The duration of Case I was 30 days, of Case II, 25 days; Dutton's case lasted for 47 days, Todd's case for 42 days. Taking such an instance as Manson's Gibraltar case and adding an incubation stage of a week, we have a period of three months and in that length of time one of these cases might travel from the remotest parts. A sporadic case of spirochetic infection may, therefore, appear anywhere at any time, and undoubtedly it occasionally so happens.

REFERENCES.

- BALDOU. *Bull. de l'Acad. Imper. de Méd.*, 1864-66, 30, p. 708.
- BIADA, M. "Un Caso de Fiebre Recurrente," *Revista de Med. y Cirug. de la Habana*, 1902, 7, p. 175.
- BILLET, A. "Un cas de typhus récurrent à Constantine," *Archv. de méd. et de pharm. milit.*, 1902, 39, p. 228.
- BRAUN, MAX. *The Animal Parasites of Man*, Sambon & Theobald, edit., London, 1906.
- BROWSE, G. "A Case of Spirillum Fever Simulating Malaria," *Brit. Med. Jour.*, 1905, 1, p. 532.
- CAMP, DE LA. "Ein Fall von Febris recurrens," *Jahrb. der Hamb. Staatskranken.*, 1893-94, Hamb. u. Leip., 1896, 4, p. 122.
- CARTER, H. V. *Trans. Intern. Med. Cong.*, 7th Sess., 1881, p. 334; *Lancet*, Lond., 1879, May 11 (note); *ibid.*, Mch. 20, 1880; *Spirillum Fever*, 8vo, Lond., 1882; "Some Recent Cases of Spirillum or Relapsing Fever," *Trans. Med. and Phys. Soc.*, Bombay, 1885, n. s., 6, p. 31; *ibid.*, 1888, n. s., 11, p. 21; *Clin. Memo of Some Cases, etc.*
- CHRISTY, C. "Spirillum Fever (Relapsing or Famine Fever)," *Jour. Trop. Med.*, 1902, 5, p. 39; "O. Moubata and Tick Fever in Man," *Brit. Med. Jour.*, 1903, 2, p. 652.
- CLEMON, F. G. *Geography of Disease*, 8vo, Macmillan, 1903.
- CLYMER, M. *Fevers: Their Diagnosis, Pathology and Treatment*, 1846, Phila.; "Notes on the History of Relapsing Fever," *N. Y. Med. Jour.*, 1870, 11, p. 55.
- COOK, A. R. "Relapsing Fever in Uganda," *Jour. Trop. Med.*, 1904, 7, p. 24.
- CROPPER, J. "Spirillum Fever in Palestine," *Brit. Med. Jour.*, 1905, 2, p. 190.
- DAVIDSON, A. *Geographical Pathology*, Edin. and Lond., 1892.
- DA COSTA, J. C., JR. *Clinical Hematology*, Phila., Blakiston, 1905.
- DEHIO, K. "Rückfallfieber," *Handb. d. Prakt. med.*, Stuttgart, 1901, 5, p. 393.
- DUTTON AND TODD. "The Nature of Human Tick-Fever in the Eastern Part of the Congo Free State, etc.," *Liverpool Sch. Trop. Med. Mem.*, 17, Nov., 1905; also abstract, *Jour. Trop. Med.*, 8, p. 244.
- ENGEL, F. "Spirochetæ Obermeieri constantirt in Egypten," *Berl. klin. Wchnschr.*, 1884, 21, p. 749.
- FAGGE, C. H. *Prin. and Pract. Med.*, Vol. 1, Phila., 1886.
- FLINT, A. *Clin. Repts. on Continued Fever, etc.*, 1852; *A Treatise on the Prin. and Pract. Med.*, 6th ed., 1886.
- FRIANT ET CORNET. "Quelques cas de fièvre récurrente dans le Dépt. de Constantine," *Archv. de méd. et de pharm. milit.*, 1904, 44, p. 421.
- GABRITSCHESKY. *Ann. de l'Inst. Past.*, 1896, 10, p. 630; 1897, 11, p. 638.
- GRAHAM, J. C. "Relapsing Fever in Sumatra," *Jour. Trop. Med.*, 1901, 4, p. 179.
- HARRIS, E. *Rept. Metrop. Health Dept.*, N. Y., 1869.
- HENDERSON, W. "On Some of the Characters Which Distinguish the Present Epidemic Fevers from Typhus," *Edin. Med. and Surg. Jour.*, 1844, 61, p. 201.
- HILL, L. G. "A Case of Spirillum Fever," *Jour. Trop. Med.*, 1904, 7, p. 35; *ibid.*, 8, p. 131.
- HIRSCH, A. *Handb. of Geog. and Hist. Pathol.*, Creighton, Vol. 1, Lond., 1883 (bibliography).

- HODGES AND ROSS. "Notes on Cases of Spirillum Fever in Uganda," *Brit. Med. Jour.*, 1905, 1, p. 713.
- HÖDLMOSEER, C. "Die Serodiagnose des Typhus recurrens," *Wien. med. Wchnschr.*, 1904, p. 2310; *Ztschr. f. Heilk.*, Wien u. Leip., 1905, 26, *Abt. f. int. Med.*, p. 506.
- JAKSCH, R. VON. *Clinical Diagnosis*, Garrod, 5th Eng. ed., 1905, p. 59.
- JENNER, W. "On the Identity or Nonidentity of the Special Cause of Typhoid, Typhus and Relapsing Fevers," *Med. Chir. Trans.*, 1850, 33, p. 23.
- KARLINSKI, J. "Zur Aetologie des Rekurrenstyphus," *Centralbl. f. Bakt.*, 1902, 31, p. 566.
- KIRK, J. "An Analysis of 150 Cases of Local Fever," *Jour. Malay Br. Brit. Med. Assoc.*, n. s., No. 1, 1903-4.
- KOCH, R. *Deutsche med. Wchnschr.*, 1905, 31, p. 1865; also abstr., *Lancet*, Lond., 1905, 2, p. 1656; also, transl., *Jour. Trop. Med.*, 1906, 9, p. 43.
- KOCH, —. "A Few Observations on Relapsing Fever" (Hong-Kong), *Med. News*, N. Y., 1905, 87, p. 1127.
- LAFFORGUE, —. "De l'existence de la spirillose humaine (typhus récurrent) en Tunisie," *Compt. rend. Soc. de biol.*, Paris, 1903, 55, p. 1132.
- LEBERT, H. "Articles on Relapsing Fever and Bilious Typhoid," *Cyclop. pract. Med. von Ziemssen*, Amer. ed., 1874.
- LÖWENTHAL, H. "Serothérapie, etc.," *Deutsche med. Wchnschr.*, 1898, 24, p. 680; also abstract, *Jour., Trop. Med.*, 1899, 1, p. 197; "Serodiagnose der Fieber recurrens, etc.," *Deutsche med. Wchnschr.*, 1897, 23, p. 560.
- MANNABERG, J. Art. on "Malarial Diseases," *Encyclop. Pract. Med.*, Nothnagel, Amer. ed., 1905.
- MANSON, P. *Tropical Diseases*, 3d ed. revised, Wood, N. Y. 1905, p. 712; "Recurrent Fever at Gibraltar," *Brit. Med. Jour.*, 1904, 2, p. 538.
- MASSEY, A. Y. "Spirillosis in Portuguese West Africa," *Jour. Trop. Med.*, 1905, 8, p. 225.
- MILLER, P. B. M. "Epidemic Relapsing Fever, etc.," *Pacif. Med and S. Jour.*, 1874-75, 17, p. 370.
- MOORE, J. W. *Textbook of the Eruptive and Continued Fevers*, 1892.
- MURCHISON, C. *A Treatise on the Continued Fevers of Great Britain*, Lond., 1873, 2d ed. (bibliography).
- MCWEENEY, E. J. "On the Relation of the Parasitic Protozoa to Each Other and to Human Disease," *Lancet*, Lond., 1905, 1, p. 785, abstr.
- NABARRO. "Spirillum Fever in Uganda," *Brit. Med. Jour.*, 1905, 1, p. 864.
- NEAL, J. B. "Relapsing or Famine Fever," *China Med. Miss. Jour.*, 1890, 4, p. 245.
- NEWSTEAD, R. "On the External Anatomy of *O. Moubata*," *Liverpool Sch. Trop. Med.-Mem.*, 17, 1905, appendix.
- NEUMANN, L. G. *Parasites and Parasitic Diseases of Domestic Animals*, Macqueen, 2d ed., 1905.
- NICOLLE ET DUCLOUX. "Note sur le typhus récurrent en Tunisie," *Compt. rend. Soc. de biol.*, Paris, 58, p. 496, and 59, p. 200.
- NUTTALL, G. H. F. "Scientific Research in Medicine," *Jour. Trop. Med.*, 1902, p. 65; *Lancet*, Lond., 1905, 2, p. 1157.
- OBERMEIER, O. *Centralbl. f. med. Wiss.*, 1873, 11, p. 145; *Berl. klin. Wchnschr.*, 1873, 35, pp. 152, 378, etc.

PLATE 6.



FIG. 1.



FIG. 2.



FIG. 3.

- PHILLIPS, L., "Five Cases of Relapsing Fever Observed in Cairo," *St. Barth. Hosp. Jour.*, 1905, 13, p. 19.
- POWELL, A. "Note on Spirillum Fever in Bombay," *Brit. Med. Jour.*, 1904, 1, p. 1014.
- RABAGLIATI AND WESBROOK. "Relapsing Fever," *Syst. of Med.*, Allbutt, 1898, 1.
- ROGERS, L. "Relapsing Fever in Kumaon Himalayas," *Jour. Trop. Med.*, 1898, 1, p. 312.
- ROSS AND MILNE. "Tick Fever at Uganda," *Brit. Med. Jour.*, 1904, 2, p. 1453.
- SAMBON, L. W. "Ticks and Tick Fevers," *Jour. Trop. Med.*, 1899, 2, p. 217.
- SANDWITH, F. M. "Relapsing Fever in Egypt," *Practitioner*, Lond., May, 1904.
- SAWTSCHENKO ET MELKICH. "Étude sur l'immunité de la fièvre récurrente," *Ann. de l'Inst. Pasteur*, 1901, 15, p. 497.
- SCHAUDINN, F. Transl., "Change of Generation and Host in Trypanosomata and Spirochætæ," *Jour. Trop. Med.*, 1904, 7, p. 171.
- SCHEUBE, B. *The Diseases of Warm Countries*, Cantlie, Lond., 1903.
- SMITH, S. *Report N. Y. Bd. of Health*, 1870.
- STRÜMPPELL, A. *A Textbook of Med.*, Vickery, 3d Amer. ed., 1901.
- TICTIN, J. *Centralbl. f. Bakt.*, 1897, 21, Abt. I, p. 179.
- United States Pub. Health and Marine Hosp. Repts., 1905, 20, Nos. 22 and 24.
- WALKER, L. A. Note on epidemic on River Indus, *Indian Med. Gaz.*, 1905, p. 320.
- WARD, G. O. "A Case of Relapsing Fever," *Boston Med. and S. Jour.*, 1900, 142, p. 36.
- WATERS, G. "Relapsing Fever, etc., in Bombay," *Glasgow Med. Jour.*, 1900, n. s., 54, p. 183.
- WELLMAN, F. C. "A Case of Relapsing Fever with Remarks on its Occurrence in the Tropics," *Jour. Trop. Med.*, 1905, 8, p. 97; also, *Amer. Med.*, 1905, p. 151.
- WOLBERG, L. "Febris recurrens u. d. Typhus bei Kindern" (Warsaw), *Jahrb. f. kinder. Heilk.*, 1887, 26, p. 222.

EXPLANATION OF PLATE 6.

These photographs (1 500 diam.) were taken from one and the same specimen of blood from Case I.

FIG. 1.—A large spirochete.

FIG. 2.—Two spirochetes seemingly end to end.

FIG. 3.—Two small spirochetes intertwined.

STUDY OF A SPIROCHETE OBTAINED FROM A CASE
OF RELAPSING FEVER IN MAN, WITH NOTES
ON MORPHOLOGY, ANIMAL REACTIONS,
AND ATTEMPTS AT CUL-
TIVATION.*

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INTRODUCTION.

THE spirochetes studied were obtained from the blood of a patient in the service of Dr. Carlisle, in Bellevue Hospital.† By inoculation of monkeys and rats we have been able to keep the spirochetes alive through the continuous passage of blood from one animal to another. It is our purpose to record as briefly as possible the various observations which we have made upon the animal reactions, with a brief description of the morphology of the micro-organism, and the various attempts made to cultivate it outside the body. Our studies have continued over a period of five months.‡

SPIROCHETAL INFECTION IN MONKEYS.

Three monkeys of the species *Macacus Rhesus*, and two of the species *Macacus Javanus*, were used for inoculation experiments. These species were selected because of their known susceptibility to infection with *Sp. Obermeieri*. The disease was produced by subcutaneous inoculation of human or animal blood containing spirochetes.

Incubation period.—Following the injection there was noted an incubation period during which no spirochetes were found in the circulating blood. The duration of this was, in one monkey (Rhesus I), two days; in another (Rhesus III), three days; in a third (Java II), three days; in the remaining two, impossible to determine, because

* Received for publication, February 7, 1906.

† Dr. Carlisle's clinical report of this case is found in this number of the *Journal*.

‡ Condensed reports of our work were given at the October and December meetings of the New York Pathological Society, and at the Society of American Bacteriologists at its December meeting of 1905, through the courtesy of Professor Novy.

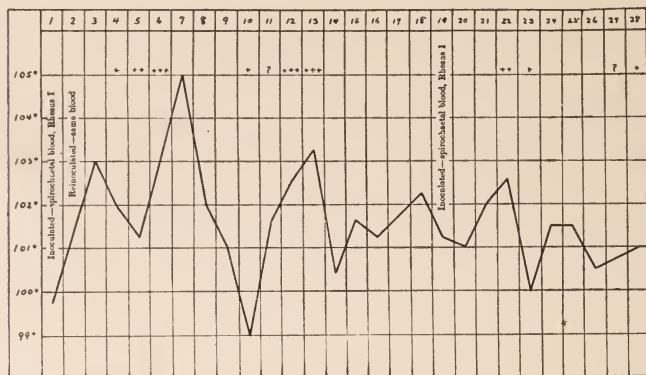
of double inoculations, but not exceeding three days in one (Java I), or six days in the other (Rhesus II). During this incubation period no obvious signs of illness were observed.

Relapses.—The disease, in monkeys, like relapsing fever in man, was characterized by a succession of paroxysms, each attended by the appearance of the spirochetes in the peripheral blood, and certain general symptoms of illness. Three of the monkeys showed three paroxysms; one monkey, four. The duration of the individual paroxysms, and of the intervals, varied widely. The longest period during which spirochetes were present in the blood on successive daily examinations was four days; the shortest, one day. The average duration of the paroxysms apparently slightly decreased with the progress of the disease. The longest interval noted was eight days; the shortest, two days. The average length of interval in general appeared to increase after the first. One marked exception to this was noted, the intervals being two, eight, and three days respectively. We see, therefore, that there is little regularity or uniformity in the onset, number, or duration of the paroxysms.

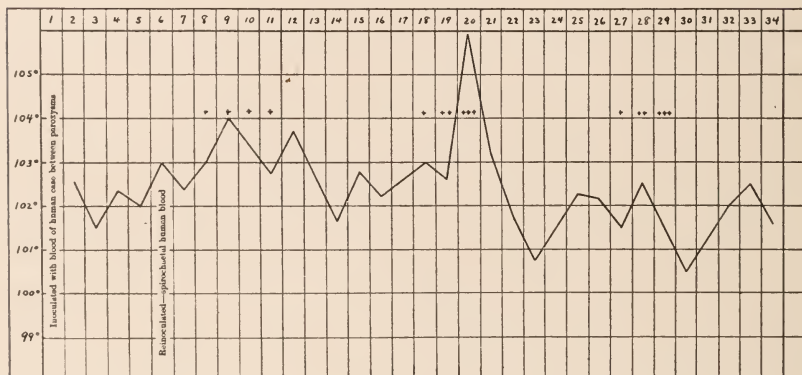
General symptoms.—A rise in temperature usually accompanies the appearance of the spirochetes in the blood, and persists until their disappearance. The temperature charts, however, are far less striking than those of relapsing-fever patients. There is normally considerable variation in the body temperature of different monkeys, and even from day to day in the same monkey. Thus Rhesus I, before the onset of his first paroxysm, showed a temperature ranging from 101° to 103° F.; Rhesus II, a temperature ranging from 99.8° to 102° ; Rhesus III, a temperature between 99.6° and 104.2° ; and Java I, between 99.8° and 102.8° . Moreover, the febrile reaction during the paroxysm, though usually present, is poorly marked and inconstant as contrasted with the typical fever curve in relapsing fever of man. It rarely exceeds 2° F. above the normal average. The highest recorded temperature is 106° , noted in Rhesus I toward the end of his second paroxysm.

The appended temperature charts show the general course of the fever. Their most impressive feature is the marked irregularity.

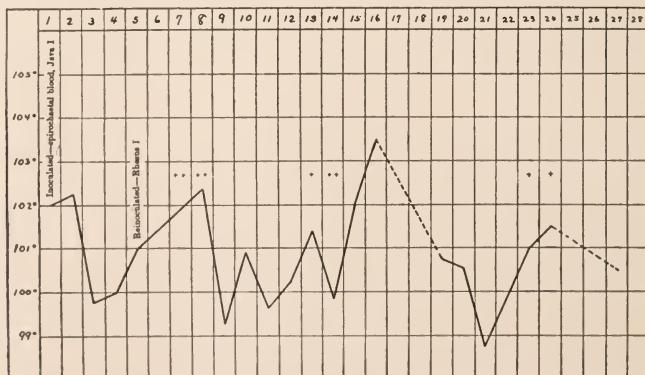
During the paroxysms the animals exhibit certain unmistakable signs of illness. When left alone, they sit huddled up against the



JAVA I.



RHESUS I.



RHESUS II.

wall of the cage, and from time to time cry shrilly, as if in pain, and, when disturbed, show an unwonted irritability. The appetite is poor during the attacks. In two monkeys there was a sharp diarrhea, lasting a few days. One monkey showed distinct, though transient, jaundice of the conjunctivæ. Edema of the eyelids was seen in Rhesus I during the latter part of his illness, and Java I developed a marked conjunctivitis of the right eye, with hemorrhage into the anterior chamber, during his second relapse.

Unfortunately, lack of time prevented us from carefully studying the blood changes during and after the paroxysms.* No differential or total leucocyte counts were made. Certain changes, however, attracted our attention in the routine examination of the blood smears for spirochetes. Very striking was the polychromatophilic degeneration of the red cells, which was constantly seen after the first appearance of the spirochetes in the blood. Granular degeneration, on the other hand, was not observed. The large mononuclears impressed us as being in excess, although without accurate differential counts, their exact numerical relation to the presence of the spirochetes in the circulating blood was not determined. Numerous very large forms, with pale, poorly staining nuclei, and ragged degenerated cytoplasm, were always present. The blood plaques appeared to be markedly increased.

The course of the disease in all monkeys was benign. The animals speedily regained their usual good health after the last paroxysm. During their illness they appeared to lose moderately in weight, especially Rhesus I and Java I.

SPIROCHETAL INFECTION IN WHITE RATS.

Up to the time of writing, about 100 rats have been inoculated. Two rats were inoculated on October 24, from Rhesus III; since this time the spirochetes have been kept alive by constant passage from rat to rat. From a few drops to 1.5 c.c. of blood, to which sodium citrate was added, was injected beneath the skin of the back. In all cases where the inoculated blood showed spirochetes at the time of injection, a spirillosis developed in the experimental animal. The only exceptions to this observation were noted in two diseased

* The blood of each monkey examined before inoculation was found free from hematozoa or other blood parasites.

rats, which died on the second day after inoculation, and showed encapsulated abscesses of lung, chronic sepsis, and advanced secondary anemia.

The incubation period ranged from one to five days, averaging about two and one-half days. It has diminished with the frequent passage from rat to rat. Thus the average time for the development of the disease in the last 15 rats inoculated was only about one day, as compared with three days in the first 15 animals used. The minimum incubation period was not determined with accuracy, because the majority of the rats were not examined until the third day. In Rat 89 numerous spirochetes were seen 19 hours after inoculation.

Owing to the fact that most of the rats were killed as soon as numerous spirochetes were found in the blood, the duration of the illness was determined in comparatively few. In those allowed to live, spirochetes were found in the peripheral circulation for a period varying from one to five days; most frequently, one to two days. It is an interesting fact that the single rat, killed after they had persisted for five days, showed multiple large chronic abscesses of the lung, from which we may infer, if we like, that his normal resistance was diminished.

No relapses were observed in two rats, whose blood was carefully studied for 12 and 18 days respectively. That relapses may not occur we cannot say definitely. The rats at no time betray any definite objective indications of illness, save the presence of the specific organisms in the blood. The same qualitative changes in the appearance of the blood cells—marked polychromatophilia, and large mononuclears, resembling bone-marrow cells, megakaryocytes—were noted as in the monkeys.

The only gross pathological change seen in animals killed during the height of infection was swelling of the spleen. No report of the histological examination of the organs will be attempted here. We must, however, state that repeated examinations of smears from the spleen, bone-marrow, and liver, stained by Wright's, Giemsa's, and other methods, failed to show spirochetes, either within or without the cells, at any stage of the disease, with the exception of a few extracellular single forms in one spleen smear. The large mononuclears, with vacuoles, we have seen in abundance. The

vacuoles contained only granular detritus; nothing in the least suggestive of spirochetes. It is possible that, with the particular organism studied, phagocytosis, if it occur at all, plays an altogether unimportant rôle. Shortly after the disappearance of the spirochetes from the circulating blood, spleen smears were likewise examined without success. This is in marked contradiction to the findings of Metchnikoff,¹ Soudakewitch,² Sawtchenko and Melkich,³ with the spirochetes of Obermeier. In splenic smears from artificially infected monkeys, spirochetes were distinctly seen and depicted within the polymorphonuclears (Metchnikoff) or the large mononuclear macrophages (Soudakewitch); in the latter they often lie coiled up within a vacuole in the cell protoplasm. Sawtchenko and Melkich observed a similar phagocytosis on the part of the endothelial cells, when spirochetal blood was injected into the peritoneal cavity of guinea-pigs.

Cantacuzène⁴ in the spirillosis of geese Levaditi,⁵ and Marchoux and Salimbeni,⁶ in the spirillosis of fowls, likewise describe the phagocytosis which occurs in the spleen toward the end of the attack. In the drawings of the spleen smears by Cantacuzène a number of clearly defined, unmistakable spirochetes are shown lying coiled within the vacuoles.

Sawtchenko, in a monkey whose spleen had previously been removed, observed that the spirochetes became very numerous during the course of infection, and persisted, a chronic spirillosis being established, the animal finally dying. The spirochetes are shown in great numbers in the blood smears taken from the superior vena cava, and within the polymorphonuclears of the peripheral blood. Such pictures were not seen during the natural course of infection in our human cases, or in the monkey or rat.* Soudakewitch² and Iwanoff⁷ noted the occurrence of phagocytosis in the circulating blood of patients with relapsing fever, near the crises, their statements varying in this respect from those made by other observers. Excision of the

* In the last edition of one of the noted American textbooks upon the clinical examination of the blood, we have been surprised to see the drawings and cuts of Sawtchenko reproduced, without any reference to their source, or any explanation that they were not drawn from human, but from the blood of the splenectomized monkey. In view of the fact that the spirochetes are never present in the blood of human being or monkeys in such abundance, and that the polymorphonuclear leucocytes in the circulating blood, according to most observers and ourselves, do not contain spirochetes, a most erroneous impression of the blood picture of relapsing fever is thereby conveyed to the reader.

spleen is not followed in all cases by a chronic spirillosis, as shown in one of our rats (90), which was splenectomized 24 hours before inoculation. In this case the infection ran the usual course, the spirochetes appearing and disappearing one day later than in the control rat.

SPIROCHETAL INFECTION IN RABBITS.

Animal Inoculated	Source of Infectious Material; Dose; How Inoculated	Remarks
Rabbit III, Sept. 19	1 c.c. of citrate blood from Java I, intravenously. (Smears positive)	Few spirochetes on following day; numerous spirochetes on second day; negative on succeeding days. No spirochetes in smears from liver, spleen, or bone-marrow
Rabbit IV, Sept. 19	0.5 c.c. of citrate blood from Java I, intravenously	Few spirochetes on following day; negative on succeeding days
Rabbit V, Sept. 21	1 c.c. of citrate blood from Rabbit III, intravenously. (Smears sp. ++)	Negative
Rabbit VI, Oct. 2	0.5 c.c. of citrate blood from Rhesus I, intravenously. (Smears sp. ++)	Negative

As seen from the above table, two of the rabbits showed undoubted susceptibility to spirochetal infection, if we are to judge by the multiplication of the spirochetes in their blood. The animals did not appear ill, and the organisms persisted for only one or two days. There were no relapses. The one attempt to inoculate a second rabbit with the spirochetal blood of Rabbit III was unsuccessful. It may be remarked here that many articles on relapsing fever state specifically that the rabbit is not susceptible to infection with the *Sp. Obermeieri*, and we have met with no previous record of successful inoculations in rabbits, except that of Dutton and Todd.⁸ This discrepancy may possibly be explained by the fact that young rabbits were used, and the material was injected intravenously. It is obvious, however, that rabbits are not well adapted for experimentation with the spirochetes, and in our further work white rats were used exclusively, because of their constant susceptibility and lesser cost.

SPIROCHETAL INFECTION IN GUINEA-PIGS.

Several guinea-pigs inoculated intraperitoneally failed to reveal spirochetes in the circulating blood.

SPIROCHETAL INFECTION IN WHITE MICE.

Two small white mice were inoculated with spirochetal blood from Rat 80. On the second day numerous spirochetes were

found in the blood. No further observations on mice have been made.

IMMUNITY.

A. *Active immunity*.—Both in rats and monkeys previous infection confers immunity. We are unable to say how long this immunity persists, since our observations cover a period of only three or four months. We do know, however, that it has lasted for 94 days in the case of Rhesus I, 86 days in Rhesus II, and 64 days in Rhesus III. Inoculations with spirochetal blood (Rat 78) after these intervals were negative. The control monkey (Java II), inoculated with the same blood from Rat 78, showed spirochetes in the blood on the third day. Only four observations have been made on rats, but those animals likewise could not be reinfected. Rats 4, 6, 41, and 46 were reinoculated after periods of 13, 10, 41 and 33 days respectively, following the last appearance of the spirochetes in their blood. Daily examinations for seven to eight days failed to show spirochetes.

B. *Passive immunity*.—In contrast to the perfect protection acquired through previous infection is the uncertainty of the protection furnished by the inoculation of serum from man and animals that have passed through a previous infection. The poor results obtained in our experiments upon the production of passive immunity are doubtless due to the fact that the serum used was not obtained from actively immunized animals—a statement in accord with the observations of Professor Novy,⁹ who found that the injection of the blood from actively immunized rats not only prevented the infection in white rats, but caused the rapid disappearance of the spirochetes from the circulating blood.

We submit a synopsis of our experiments along this line, and the inferences which in our opinion may reasonably be drawn from them:

a) In these experiments serum and spirochetal blood were mixed before inoculation.

Experiment I.—Rat 22: Inoculated with 1 c.c. of blood from Rat 16, and 1 c.c. of normal human serum (kept five days at room temperature); in contact for five minutes before inoculation. Spirochetes appeared on the third day in considerable numbers.

Rat 24 (control): Inoculated with 1 c.c. of blood from Rat 16. Spirochetes appeared on the third day.

Rat 21 (control): Inoculated with 1 c.c. of blood from Rat 16. Spirochetes appeared on the third day.

Normal human serum exerted no inhibitory power upon the development of the infection.

Experiment II.—Rat 23: Inoculation with 1 c.c. of spirochetal blood from Rat 16 + 1 c.c. of human serum (A.M.P.) obtained 12 days after second and last relapse. (Serum kept for five days at room temperature.) Spirochetes appeared on the fifth day.

Rats 21, 24 (controls): See Experiment I.

The "immune" human serum apparently caused a retardation of the infection for two days.

Experiment III.—*Rat 25: Inoculated with 1 c.c. of blood from Rat 16 + 1 c.c. of serum from Rhesus I, obtained 43 days after the third relapse, and 15 days after inoculation with spirochetal blood from Rat 1. (Serum kept 24 hours at room temperature.) Blood negative for nine days; not further examined.

Rats 21, 24 (controls): See Experiment I.

Complete protection was afforded in this case by the serum of a monkey after natural infection, and after a single subsequent inoculation with spirochetal blood.

b) In the following experiment the immune serum was injected previous to the inoculation with infective blood.

Experiment IV.—Rat 26: Inoculated subcutaneously with 20 gtt. of serum from Rhesus I, drawn 43 days after the last relapse, two days old. Three days later inoculated with 15 gtt. of spirochetal blood from Rat 21. Spirochetes appeared on the third day.

Rat 30 (control): Inoculated with spirochetal blood from Rat 21. Spirochetes appeared on second day.

c) In the following experiment an unsuccessful attempt was made to cause the disappearance of the spirochetes from the blood during a paroxysm, by the injection of the citrate blood of a rat which had passed through its infection. We were led to perform this experiment by the very striking and favorable results recorded by Professor Novy⁸ in the use of blood from *actively* immunized rats.

Experiment V.—Java II: Inoculated with spirochetal blood from Rat 78. A few spirochetes appeared on the third day, and were very numerous and extremely

* A similar set of experiments was performed five days previously. The results are not given in detail, because two of the rats escaped from their cages, and could not be positively identified. In this case the immune human and monkey serum afforded protection, whereas control rats and rat inoculated with normal human serum developed spirochetes on the third day.

motile on the fourth day. On the fourth day the monkey was inoculated subcutaneously with 5 c.c. of blood of Rat 75, withdrawn five days after last appearance of the spirochetes. Blood was drawn from the ear and examined every 15 minutes for one hour, and again after two, three, and five hours.

No effect was produced, the spirochetes becoming more numerous and retaining their motility after five hours. On the following day, 24 hours later, no spirochetes were found. The first relapse occurred five days later.

It is evident from this experiment that the anti-bodies in the blood of a rat which has recovered from the spirochetal infection are not present in quantities sufficient to abort the infection in other animals. Furthermore, the inoculation of this serum did not inhibit or retard the onset of the first relapse, or shorten its duration.

It may be of interest to note an additional observation made at this time. Immune blood of the above rat (No. 78), as well as the immune blood of Rhesus III, when mixed with the spirochetal blood of Java II, and examined in hanging-drop, showed no agglutinating properties, nor was any decrease in the motility of the organisms observed, after several hours. (Apparent lack of "immobilisines" of Levaditi.) The only phenomenon noted was a marked tendency of the spirochetes to adhere to the red blood corpuscles. In the hanging-drop preparation, after 24 hours at room temperature, no spirochetes were found, however, whereas in the control hanging-drop, and with mixtures of Java II and Rat 75, numerous clumps of spirochetes were seen, only a few of the organisms being still motile. The dissolution or cytolysis of the spirochetes evidently took place slowly in serum.

MORPHOLOGY.

Because of the emphasis that has been laid upon the morphology of the spirochetes as a basis for their classification, by Schaudinn¹⁰ and other writers, it seems worth while to enter into a somewhat minute description of the forms which the organisms studied by us may assume.

Under conditions of which we know practically nothing, these spirochetes may show the most astonishing variations—variations in length, in thickness, in the number and closeness of the spirals, in motility, and in arrangement.

The spirochete, as seen in smears, is a tapering, spiral filament, whose length varies roughly from one and one-half to 10 times the

diameter of a red cell; exceptionally, even longer forms are seen. The breadth or thickness is more constant; but still one meets with very slender (attenuated) forms, on the one hand, and thick, almost ribbon-like or fusiform types, on the other. In some rats we have observed that the breadth varies markedly in different portions of one and the same spiral. The number and conformation of the spirals are subject to very great variations. The conditions which determine these variations in morphology we have not been able to ascertain positively. These marked variations are observed only when the spirals are most numerous; that is, within 24 hours previous to their disappearance from the circulating blood. The extremes are typified by a short organism, showing but a single sinuous curve; and a long one with 15 or more closely approximated spirals. Between these two there may be found every transitional form.

The tapering ends of the spirochetes in successful smears are usually drawn out to a considerable length, so that they may readily be interpreted as terminal whips or cilia. In special stains for cilia we have never seen more than one terminal filament.

In every stained preparation examined the majority of the spirals show in their central portion an unstained area, where the substance of the spirochete is of smaller caliber, as if it were thinned out. In long forms, two, occasionally three, attenuated portions may be seen. This attenuation may be so marked at one point that it is difficult to say whether the continuity between the two portions is preserved or broken. Numerous spirochetes may also be found, lying end to end, with their tapering extremities in close approximation, strongly suggesting a recent separation.

These appearances, to our mind, force the conviction that reproduction in these spirochetes normally occurs by transverse fission, or possibly fragmentation. In spite of painstaking study, we have not succeeded in actually seeing this take place in living preparations. Because of their active motility, their low refractive index, and the difficulty in keeping the entire spiral in one optical plane, it is practically impossible to watch the same organism continuously for any length of time.*

* On one occasion transverse division was observed, but, owing to the difficulty of being positive in the interpretation of such phenomena, the above conservative statement is advisable.

In stating that the spirochetes multiply by transverse division, we do not exclude the possibility that other modes of reproduction may take place. Of longitudinal division, or of sporulation, we have seen no evidence.

When the spirochetes are abundant, one frequently finds two or more organisms entwined, or, when very numerous, in tangled masses. They are, however, so often seen in certain definite relations to each other that one might be inclined to attribute to this a more than casual significance. We have been impressed with the frequency with which two spirochetes are seen entwined throughout their entire length, or lying side by side, accurately apposed, or even apparently fused through a portion of their length. The fused portions may terminate as a broad mass, ending in several short terminal filaments. We do not think that this appearance signifies a longitudinal division. These appearances remind one of the fusion which has been observed in the bacteria; or, again, it may be simply an expression of their tendency to adhere to each other under certain conditions. The spirochetes tend to adhere to the red blood cells, to the hemokonia or blood dust of Müller, to the blood plaques, and to various particles present in the blood. Finally, as a theoretical possibility, one may think of a conjugation analogous to that seen in the protozoa.

Extraordinary forms have been noted in the blood of a few rats, which require notice. In Rat 87 the spirochetes were found in great numbers. Many of the tangled masses were composed of individuals having the usual spiral appearance. Other masses were composed of more or less flattened or ribbon-like, and partly sinuous or even straight, individuals, these varied forms being more or less intimately united by faintly staining intercellular substance. Ribbon-like forms were quite common. In some of these thick forms an intervening clear space was made out, with the aid of compensation ocular No. 8. (See Plate 7, Fig. 4.) The ribbon-like forms may stain quite intensely, although usually they show a pale-blue color with the ordinary blood stains. Forms are not infrequently seen in which most of the protoplasm stains faintly, with several more deeply stained areas. The appearance occasionally suggests the presence of a macro- and micro-nucleus, but, on the whole, the

more acceptable, and less strained, explanation is that, as in bacteria, irregularity in staining occurs. To us the ribbon-like forms are analogous to the so-called involution or degeneration forms of bacteria. The peculiar forms above described we have seen in rat blood, when the spirals are numerous, just before their final disappearance from the circulating blood. Under such conditions, it seems more reasonable to infer that these peculiar forms are the expression of degenerative or necrobiotic changes rather than developmental changes or evidences of longitudinal division.

STAINING REACTIONS.

Films have been stained with Wright's, Jenner's, Giemsa's, and Goldhorn's blood stains; with the ordinary aniline dyes, and with Gram's stain, the spirochetes decolorizing by the latter method; also with Loeffler's and with Proca's¹¹ flagella stain.

Wright's stain has been used for routine examination. With this stain the spirals are colored a blue of moderate intensity, or, if the differentiation is carried farther, a faint or even a pronounced red. The other polychrome stains have yielded, in our hands, no essentially different results. Hematoxylin stains them faintly, but uniformly.

With methylene blue, followed by Gram's iodine, the spiral is stained grayish black throughout its entire length, this being the color which the metachromatic granules of bacteria assume when stained by this method. The results obtained by the use of these stains—that is, a uniform, diffuse stain—speaks strongly against the assumption of the presence of a macro- and micro-nucleus. By means of aqueous methylene blue an intra-vital staining may be brought about in the hanging-drop, the spirals retaining their motility for 24 hours or longer.

MOTILITY.

In fresh hanging-drop preparations the most characteristic movement is a very active wavy or corkscrew one, broken by an asystolic interval of variable length.* This movement may apparently start from either end, and an alternation in the wave of contraction is always noted, except when a spiral becomes attached by one end

* For very simple optical reasons, it is impossible to decide whether this is an actual revolution about the long axis, or a rhythmic waving in one plane, comparable to the waving of a pennant in the wind.

to some object. A very definite lateral oscillation also takes place. This ranges from a slight trembling to a violent to-and-fro agitation of the entire spiral. At times a distinct and rapid forward-and-backward progression is seen, the total excursion sometimes covering half the microscopic field of an immersion lens.

In the hanging-drop kept for two days, either at room temperature or in the incubator, the spirochetes retain their various forms of movement. As their vitality becomes impaired, however, their movements grow less rapid, and often assume a tetanic character. When the corkscrew movements become slow, and occur at longer intervals, it can readily be made out that the motion in one direction originates at one extremity, the other extremity determining motion in the reverse direction.

DEGENERATIVE AND NECROBIOTIC CHANGES.

With this gradual loss of motility the spirochetes undergo a marked change in appearance. The threads become swollen and unevenly refractive, resembling the thick, ribbon-like forms found in stained blood smears. Later there are constantly found tenuous, beaded forms, which may be compared to some of the irregularly staining or moniliform individuals seen in the blood films. The presence of such forms only shortly precedes the final disappearance of the spirals from the citrated blood.

It is our impression, therefore, that these forms represent a stage in the dissolution of the spirochetes.

ATTEMPTS AT CULTIVATION.

Many attempts have been made to obtain cultures of the spirochetes, all of which have been unsuccessful. Basing our choice of media upon a possible relationship between these organisms and the trypanosomata, the 2 to 1 rabbit blood agar as recommended by Novy and McNeal¹² was tried. This medium, though tested a number of times, gave no results.

Various attempts were made to obtain cultures in fluid media. These media may be briefly mentioned: human blood with meat extract broth; human blood with human muscle broth; human blood with rabbit muscle broth; ascitic broth with and without blood. Inoculations on these media were negative. In some cases the blood, before being added to the broth, was mixed with sodium

citrate solution, and in others the medium containing blood was heated for one-half hour at 55°C ., to destroy the complement.

In some cases the blood was laked by citric acid, and subsequently neutralized by the addition of sodium carbonate.

Cultures were kept both at room temperature and in the incubator. In none of these fluid media was any evidence of multiplication observed, the spirals never being recovered.

Evidences of multiplication, however, were obtained in the condensation water of human and rat blood agar, the agar being made up with 2 per cent peptone and 1 per cent dextrose, and with human muscle infusion instead of the usual Liebig's meat extract. The condensation water, after 24 hours at room temperature, showed actively motile spirochetes in the hanging-drop, in greater numbers than in the original blood, as determined by the number seen in smears of the blood made at the time of inoculation. Transplants of a few loops from the condensation water to similar blood-agar tubes yielded practically negative results, although in many cases a few spirals were found on examination. The tubes kept at 37°C . uniformly gave negative results.

EVIDENCES OF MULTIPLICATION OF THE SPIROCHETES IN HUMAN AND RAT CITRATE BLOOD AT ROOM TEMPERATURE.

Positive evidences of the multiplication of the spirochetes in fluid media were observed in a number of experiments. In human and rat blood, to which has been added sodium citrate to prevent coagulation, there can be seen, within 24 hours after inoculation with a few drops of rat blood containing spirochetes, a very evident increase in the number of these organisms. By this we mean that, notwithstanding the dilution of several drops of infected blood with 60 to 150 times its volume, the spirochetes are more numerous in the smears from the culture fluid than in control smears taken at the time of inoculation.

Furthermore, after inoculating several drops of this first generation into a second blood tube, we have found the organism in approximately the same numbers in the second transplant.

The third generations, however, have failed to grow, although in the hanging-drop, non-motile, and in stained specimens, granular or moniliform organisms were seen. Although multiplication of

these organisms in citrate blood was the rule, in a few cases increase was not observed. For instance, citrate blood, obtained from some patients, seemed to exert a deleterious influence upon the spirochetes, none being found in the blood after 24 hours. These variations in human blood may account for some of the negative observations. Multiplication likewise occurs in the undiluted citrate blood from infected rats, kept over night at room temperature, and in many cases tangled clumps develop.

The following brief description must suffice to justify the above statements upon the occurrence of multiplication:

Rat 51: December 4, inoculated with citrated blood of Rat 49, showing spirochetes. December 7, a few spirochetes found in stained smears. Rat was then etherized, and blood aspirated from heart. A few drops of blood introduced into a test tube containing 5 c.c. of human citrated (circa 3 per cent sodium citrate) blood. Tube kept at room temperature. In hanging-drop preparations, 19 hours after inoculation and in smears stained by Wright's stain, numerous single as well as tangled masses of spirochetes were seen. The drops for examination were taken from the surface of the somewhat turbid supernatant fluid. Transplants of a few drops from this tube into human citrate blood, on examination after 24 hours at room temperature showed actively motile spirals, not as numerous as in the first generation. Third generations were practically negative.

An agar flask containing 20 c.c. of Medium H* and 2 c.c. human citrate blood inoculated with a drop of infected rat blood, and kept for 19 hours at room temperature, showed numerous spirochetes in hanging-drop.

Rat 54: December 6, inoculated with blood of Rat 50, latter showing a few spirochetes. December 9, a few spirochetes found in smears made from blood of rat's tail; rat etherized, heart's blood aspirated, and mixed with citrate solution, and a tube containing 3 to 5 c.c. citrate human blood was inoculated with two drops of blood. After 24 hours at room temperature, the rat blood diluted with citrate solution showed numerous single and tangled masses of spirals in hanging-drop and in smears from fluid taken from surface of the settled blood tube. Transplants from this tube in sterile citrate rat blood were unsuccessful. In the citrate human blood tube no spirals were found. Plain human meat broth, and two tubes of human meat broth 5 c.c. + 1 c.c. human citrate blood, inoculated with several drops of blood of Rat 54, show no spirochetes on examination.

In this experiment there was an evident multiplication of the spirals in the original rat blood, no multiplication being observed in the dilutions.

Rat 55: As in Rat 54, there was an evident multiplication of the spirals in the original citrate rat blood after 24 hours at room temperature. No evidence of growth occurred in the citrate human blood tubes inoculated with a few drops of rat blood.

Rat 56: December 9, inoculated with blood of Rat 54, latter showing numerous

* Medium H:

Peptone.....	2 per cent
Sodium chloride.....	5 "
Dextrose.....	2 "
Agar.....	2 "
Slightly alkaline to phenolphthalein.	

spirals. December 11, extremely numerous spirals were found. Citrate human blood (3-5 c.c.) tubes, and normal rat citrated blood (3-5 c.c.), were inoculated with several drops of the heart's blood of Rat 56, and the rest of the blood was citrated and kept at room temperature. After 18 hours the spirochetes had multiplied greatly in the original citrated blood (Rat 56). Many large clumps and numerous single spirals were seen in the hanging-drop and in stained smears made from the fluid. The normal rat and the normal human blood likewise showed extremely numerous and actively motile spirochetes. The spirals were found not only on the surface, but also in equal numbers at different levels of the fluid, proved by carefully drawing off the fluid in fine capillary pipettes. Transplants to normal rat blood showed in hanging-drop a few slightly motile, but beaded spirals. Transplants from this second generation remained negative.

This experiment indicates that in the first generation in normal rat blood rapid multiplication takes place, but that further growth is for some reason inhibited. It has been constantly observed that where laking of the citrated blood occurs, as in the second-generation tubes of this experiment, no multiplication takes place.

Rat 57: December 9, inoculated with blood of Rat 54. December 12, a few spirals found in one large smear. Rat etherized, blood citrated, and a few drops inoculated into human citrated blood. After 18 hours the spirochetes were found in considerably greater numbers in the culture tubes than in tubes containing the original citrated blood.

Transplants of a few drops to tubes of the same medium showed, after 24 hours, a few motile and non-motile spirals in hanging-drops and in smears. Transplants from these tubes were unsuccessful.

Rat 58: December 11, inoculated with blood of Rat 57. December 14, spirochetes found. Smears of the blood at time of etherization of rat showed two spirochetes after five minutes' search. Rat etherized, and blood inoculated into tubes, which, 24 hours later, gave the following results:

Tube 1, normal citr.	rat blood, inoc. with 2 gtt.	Sp. not found.
" 2, " " "	" " " " 2 "	Sp. moderate number.
" 3, " " "	" " " " 2 "	Sp. numerous.
" 4, " " "	" " " " 2 "	Sp. few.
" 5, " " "	" " " " 4 "	Sp. not found.

(heated 30' at 56° C.)

Tube 6, normal citr. rabbit blood, inoc. with 4 gtt. Sp. not found.

Transplants were made as follows:

From tube 3: a few drops into	{ citrated rabbit blood—2 tubes
	{ " rat " 1 tube
From tube 2: a few drops into	{ citrated rabbit blood—2 tubes
	{ " rat " "

After 24 hours at room temperature, transplants from Tube 2 showed a few straight, typical, slightly motile and non-motile forms; while the original citrated blood (Rat 58), examined on the same day (48 hours), showed numerous active spirals. Transplants from Tube 2 negative.

The extreme scarcity of the spirochetes in the blood at time of killing of rat, when compared with numerous spirals found in the above blood tube at 48 hours, as well as in transplants, indicates active multiplication. In this experiment the rapid multiplication observed is confined to the tubes of the first generation. Something inimical to the growth and even preservation of the spirochetes makes its appearance.

Rat 59: December 11, inoculated with blood of Rat 57. December 14, spirochetes numerous. December 15, spirochetes numerous, rat etherized, several drops of blood inoculated into:

3 tubes of normal citrated rabbit blood.
 3 " " " " " rat " "

Cultures on the three rabbit blood tubes were negative, when examined 24 hours later. A moderate number of spirals were found in hanging-drops from the rat blood, and in one of these tubes (Tube 6; see Plate 7, Fig. 6), clumps of actively motile spirochetes were found after 24 hours. Several loops of this tube, transplanted into citrated rat blood, revealed six actively motile spirals in one hanging-drop, which, when the dilution is taken into account, may indicate only the number actually carried over.

Rat 64: December 17, inoculated with blood of Rat 62, latter showing numerous spirochetes. December 20, very numerous single spirochetes, and also small clumps found in stained smears.

Cultures made on three tubes of citrated normal rat blood, each tube being inoculated with one drop of spirochetal blood. Tubes examined after 24 hours at room temperature.

Tube 1, a few motile spirochetes found.
 " 2, no spirochetes found (slight laking).
 " 3, not examined

Tubes after 48 hours at same temperature:

Tube 1, spirochetes in moderate numbers.
 " 2, only one spirochete found in hanging-drop.
 " 3, spirochetes in moderate numbers.

This experiment has been introduced for the purpose of demonstrating that the best cultural results (indicated by multiplication) are obtained with the blood of rats which show few spirochetes. (See above experiment, Rat 58.)

Considering the large numbers of spirochetes in the blood of Rat 64, no multiplication can be claimed in this experiment, even when the dilution of the blood is taken into account (1 drop in 3-5 c.c. of normal citrate rat blood).

Rat 66: December 20, inoculated with blood of Rat 64, showing spirochetes very numerous. December 22, spirochetes in moderate numbers. Cultures in normal citrate rat blood were negative, while spirochetal rat blood after standing 24 and 48 hours, showed numerous spirochetes, most of them motionless and in clumps.

Rat 83: January 8, inoculated with blood of Rat 81. January 10, a few spirochetes found in blood after search through many fields. Rat etherized, and two drops of blood inoculated into 5 to 6 c.c. of citrated normal rat's blood. Blood of Rat 83 citrated and distributed in two tubes. After 24 hours at room temperature, the three tubes showed extremely numerous and actively motile spirochetes, a dozen or more to the field of the hanging-drop, and after 48 hours showed clumps and spirochetes which had lost their motility. Transplants (two loops), made after 24 hours upon the same medium, show next day only a few spirochetes.

This experiment indicates not only that a multiplication of the spirals must have occurred in the original citrate rat blood, but that a much greater multiplication took place in the normal citrated rat blood inoculated with only two drops.

As an illustration that this multiplication by no means always occurs in the normal rat blood, the following experiment is given:

Rat 85: January 10, inoculated with blood of Rat 83. January 12, a few spirochetes found. Rat etherized. The citrated blood, and the normal citrated rat blood inoculated with a few drops, were examined 24 hours later. Only an occasional spiral was found in the citrated blood of Rat 85, the dilution being negative at this time and also later.

We believe that from these experiments we are justified in concluding that under favorable conditions, a multiplication of the spirochetes occurs outside of the body. This conclusion is based upon the experiments detailed above, the results of which may be summarized as follows:

If a few drops of spirochetal rat blood be inoculated into 3 to 5 c.c. of citrated blood (human or rat blood), the spirochetes are frequently found in greater numbers than in the original infected rat blood kept, under similar conditions, at room temperature; and in much greater numbers than in the circulating blood when the tubes are inoculated, since at this time their discovery often requires a prolonged and careful search.

That the spirochetes found in the fluid medium represent the individuals which have been transferred with the few drops of rat blood is opposed by the fact of their being more numerous than in the original citrated rat blood, and also by the fact that they are present in approximately equal numbers at different levels of the fluid.

Only three previous observers have noted an increase in the numbers of spirochetes under artificial conditions.

Multiplication in defibrinated blood was noted by Lachmann¹³ in 1880. Three experiments illustrating this are cited by him. Whereas examination of the blood when withdrawn (at the onset of the symptoms) showed spirochetes to be either absent or present only in small numbers, they appeared to increase rapidly in the blood removed, so that on the following day 3 to 20 were found in each field.

Albrecht¹⁴ also states that he has frequently observed multiplication, but gives no detailed observations. His account of the development of the spirochetes *in vitro*, from refractile particles in the blood, has not been substantiated and is doubtless incorrect.

Gerhardt¹⁵ has also noted an increase in numbers in blood withdrawn during a paroxysm, as well as their appearance after 24 hours in blood taken just before the onset of the attack, at a time when no spirochetes were found.

All other observers, on the other hand, have denied the occurrence of this multiplication. (See Kolle u. Wasserman.¹⁶)

GENERAL CONSIDERATIONS UPON THE IDENTITY AND THE BIOLOGY OF THE SPIROCHETES ABOVE DESCRIBED.

A discussion and review of the literature of this class of micro-organisms, the spirochetes, would extend the limits of the article unnecessarily. Moreover, this topic has been excellently and fully presented by Wladimiroff,¹⁷ and quite recently by Waldemar Löwenthal,¹⁸ while references to the recent articles are found in the publication edited by Schaudinn.¹⁹

However, a few words may not be amiss in calling attention to the recognition of a certain number of new diseases in man and animals as being caused by species of this interesting class of micro-organisms. Twenty-three years after the demonstration of the spirochete of relapsing fever by Obermeier²⁰ (1868; observation published 1873), Sakharoff²¹ (1891) demonstrated the etiological connection of *Spirocheta anserina* with septicemia of geese. Marchoux and Salimbeni,⁶ in 1903, published their observations upon the Brazilian disease of fowls, and have likewise established another spirochete, the *Sp. gallinarum*, as the pathogenic agent of this disease. Laveran,²² in 1902, announced, for A. Theiler, the discovery of a spirochetal infection of cattle in South Africa, due to the *Sp. Theileri*. Martoglio and Carpano²³ have recorded a case of spirochetal infection in a sheep, the organism being essentially similar in morphology to the spirochete of Obermeier. By the observations of Ross and Milne,²⁴ Dutton and Todd,⁸ of R. Koch,²⁵ and of other observers, the tick fever of man in Africa has been definitely decided to be due to a spirochete which greatly resembles in its morphology the *Sp. Obermeieri*.

C. Nicolle and C. Comte²⁶ describe briefly a spirochetal infection of bats in Tunis, which is of especial interest on account of the prevalence of relapsing fever in the Arabs of Tunis and the surrounding country. Monkeys and white mice are immune. In morphology it resembles, according to these observers, the spirals of relapsing fever. It has finely tapering extremities, stains easily, and divides by transverse division. All these spirochetal blood infections have one characteristic in common, namely, the constant presence in the

circulating blood of the spirochetes during the course of the disease.

The morphology of these various spirochetes, according to different observers, is again strikingly similar. Thus, the description of *Sp. Theileri*, of *Sp. gallinarum* by Levaditi, of the spirochetes found in bats by Nicolle and Comte, of those found in sheep by Martoglio and Carpano, and the spirocheta of tick fever described by R. Koch, are in striking accord, and are not at variance with the morphological appearances presented by our spirochete. At the present time it is only by means of the animal reactions produced by this class of microorganisms that those producing the infections in man can be separated from those causing infections in animals. With these points in view, the *Sp. anserina* may be considered to be a species distinct from *Sp. gallinarum*.

While Dutton and Todd believe the relapsing fever of Europe and the tick fever of Africa to be identical, Koch has observed certain differences, such as the mildness of the clinical symptoms, and scarcity of the spirals in the circulating blood. As a result of his researches with the spirochete isolated by us, Professor Novy⁹ believes that it is identical with the *Sp. Obermeieri*. Furthermore, he is inclined to believe that the relapsing fever of Europe and the tick fever of Africa are due to different spirochetes, on account of differences in the course of the spirochetel infection in white rats, as observed by Dutton and Todd, and by himself.

In contrast to the benign disease brought about in monkeys by the inoculation of our spirochete, the observations recorded by Dutton and Todd, and by R. Koch, show that the infections in their monkeys ran a more severe, or even fatal, course.

Infection in our monkeys has been constantly attended by the appearance of several paroxysms, while the disease caused in the same animals by the *Sp. Obermeieri* is characterized by the rarity with which relapses occur. Since inoculations in white rats with the latter organism have not been recorded, the infections of these animals described by Dutton and Todd, and by ourselves, cannot be claimed to be differential animal reactions.*

* Several observers (Carter,²⁸ Karlinski²⁹) state that mice are insusceptible: whereas Mamurowski³⁰ states that rats are naturally immune.

The question of the hour concerns itself with the classification of the spirochetes. Schaudinn believes that *Sp. pallida* and all other spirochetes are protozoa, and not bacteria. Vuillemin²⁷ has renamed the *Sp. pallida*, designating it *Spironema pallidum* on account of certain morphological appearances. This nomenclature was at first accepted by Schaudinn, but later, owing to the fact that the name *Spironema* is used in zoölogy for another form of life, he proposed the generic name *Treponema*.

According to Schaudinn, the coils of the *Sp. pallida* are permanent—a characteristic which serves, with the demonstration of a terminal cilium, to separate it from the true spirochetes, which have more or less blunted ends. The *Sp. pallida* is differentiated by Schaudinn from the spirilla by the presence of only one cilium instead of a terminal bush of cilia, by the flexibility of the spirals, and by the occurrence of longitudinal division. Although he considers this last point not positively established, nevertheless he believes the occasional demonstration of two terminal cilia to be an indication of approaching longitudinal division. He has been able to demonstrate in the living and in stained specimens a membrane or a periblast in all species of spirochetes, except the *pallida*. Lately, he is under the impression that he has detected a membrane around *Sp. pallida* in the fresh condition.

Following Schaudinn's argument, our spirochetes must be classed with *Sp. pallida*. The ends are invariably drawn out, and terminate in delicate filaments, quite as long and as delicate, if not more so, than the cilia he has described and depicted for the *Sp. pallida*. These thinned-out ends he terms cilia. But, as we have stated above, these terminal attenuations differ materially from the cilia of bacteria, since, unlike the latter, they appear to be direct prolongations of the body protoplasm, and, in accordance with this, have the same qualitative staining reactions. For these reasons, it seems to us more appropriate to call the tapering extremities of our spirochetes, as well as those of *Sp. pallida*, terminal filaments.*

* Since writing the above section, there has appeared in the *Berl. klin. Wchnschr.*, 1906, No. 7, Feb. 12, a further communication from R. Koch, relating to the African recurrent fever. In this article Koch states that he has been unable to confirm the statements of Schaudinn upon the *Sp. Obermeieri*. He, like ourselves, has found no evidence of a blepharoplast, nucleus, or undulating membrane (Flimmer-saum). Professor Zettnow, to whom Koch has referred for support concerning the morphological evidences on these points, has made the interesting observation that the terminal filament of the spirochetes unlike the cilia of bacteria, stain with methylene blue—an observation which confirms our own.

Furthermore, our spirochetes in rest and in motion retain their spiral coils.

We have already referred to our photographs which show the closest approximation to an indication of longitudinal division which we have observed in many hundreds of stained smears of rat, monkey, and human blood. It is not necessary to enter again into a discussion of our explanation of such appearances.

On account of the unsatisfactory evidence which the study of the morphology of this class of microorganisms has furnished, many investigators have made attempts to determine the nature of the spirochete by a study of its biological characteristics in those insects which are known to be the active agents in the transmission of the various spirochetal infections. The first observations seem to have been made by Tictin³¹ upon the transference of relapsing fever to monkeys, by the injection of the crushed bodies of bed-bugs which had fed upon the blood of fever patients. These observations prove simply that the spirochetes remain alive a few days in the bed-bug.

Marchoux and Salimbeni have infected fowls by the bites of certain insects, the *Argas miniatus*, which have fed on spirochetal blood. Theiler believes that the African cattle fever is transferred by ticks under natural conditions. Dutton and Todd have lately shown that the tick is responsible for the transmission of the human tick fever. Ticks developed from eggs dropped by infected insects transmit the spirochetal infection to monkeys. These observations, and those made by Schaudinn upon the developmental stages of *Sp. Ziemanni*, coupled with the weight of his authority, have induced many to regard the spirochetes as protozoa. A careful examination of Dutton and Todd's paper shows that they have observed no developmental stages in the tick, which would justify them in their conclusion that the spirochetes undergo a cycle. They found the spirochetes unchanged in the stomach and in the malpighian tubes. On the other hand, Koch, it seems, has definitely shown that they undergo no developmental stages in the tick, but pass out of the stomach in a few days, and reach the ovary, where they multiply rapidly, and surround the eggs in tangled masses.

Borrel and Marchoux³² have shown that the *Sp. gallinarum* remains alive and infective for a long time in the bodies of the *Argas*

miniatus, when the insect is kept at a temperature of 35° C. The spirochete is found at first only in the stomach; later it pierces the stomach wall, and may be found in the general body cavity and in the ducts of the salivary glands. No evidence was obtained of a developmental cycle or transformation of the spirochetes within the bodies of this insect. These observations are in accord with the well-known viability of the *Sp. Obermeieri*, as shown by its remaining alive in capillary pipettes and in blood kept at room temperature for several days. Pasternazki,³³ fifteen years ago, showed that the spirochetes remain alive in the body of the leech for a considerable period of time, and, more recently, Karlinski³⁴ has found them alive in bed-bugs for thirty days after feeding on spirochetal blood.

The foregoing considerations as to the biological status of the spirochete, based upon our work and that of other observers, appear to warrant the following statement:

The absence of any definite indication of longitudinal division; the absence of such chromatic particles as justify belief in the existence of macro- and micro-nucleus; the unquestionable occurrence of transverse fission; the positive evidence of the production of active immunity, and, as shown by Professor Novy, the formation of antibodies in sufficient amount to lend passive immunity: these facts, the authors believe, indicate that the spirochetes must not be considered as protozoa.

On the contrary it would seem more rational, at the present time, to consider them to be either bacteria or in a class by themselves.

LITERATURE.

1. METCHNIKOFF. *Virch. Arch.*, 1887, 109, p. 176.
2. SOUDAKEWITCH. *Ann. de l'Inst. Pasteur*, 1891, 5, p. 545.
3. SAWTCHENKO AND MELKICH. *Ibid.*, 1901, 15, p. 521.
4. CANTACUZÈNE. *Ibid.*, 1899, 13, p. 529.
5. LEVADITI. *Ibid.*, 1904, 18, p. 129.
6. MARCHOUX AND SALIMBENI. *Ibid.*, 1903, 17, p. 569.
7. IWANOFF. *Centralbl. f. Bakt.*, 1897, 22, p. 117.
8. DUTTON AND TODD. *Lancet*, Aug. 5, 1905; *Jour. of Trop. Med.*, 1905, 8, p. 244; *Liverpool School of Trop. Med.*, 1905, Memoir 17.
9. NOVY AND KNAPP. *Jour. Am. Med. Assoc.*, 1906, 46, p. 116.
10. SCHAUDINN. *Deutsche med. Wchnschr.*, 1905, 31, p. 1665.
11. PROCA. *Compt. rend. de la Soc. de biol.*, 1905, 58, p. 1044.

12. NOVY AND MACNEAL. *Jour. Infect. Dis.*, 1905, 2, p. 265.
13. LACHMANN. *Deut. Arch. f. klin. Med.*, 1880, 27, p. 526.
14. ALBRECHT. *Ibid.*, 1881, 29, p. 77.
15. GERHARDT. *Verhandl. d. phys.-med. Gesell. in Würzburg*, 1881, No. 2.
16. KOLLE UND WASSERMANN. "Rückfallfieber," *Handb. d. path. Mikroorg.*, Jena, 1904, p. 1126.
17. WLADIMIROFF. See Kolle und Wassermann.
18. LÖWENTHAL. *Deutsche med. Wchnschr.*, 1897, 23, p. 560.
19. SCHAUDINN. *Archiv für Protistenkunde*.
20. OBERMEIER. *Virch. Arch.*, 1869, 47, pp. 161, 428; *Centralbl. f. med. Wiss.*, 1873, 11, pp. 145, 561; *Berl. klin. Wchnschr.*, 1873, pp. 152, 378, 391, 406, 455.
21. SAKHAROFF. *Ann. de l'Inst. Pasteur*, 1891, 5, p. 564.
21. LAVERAN. *Compt. rend. de l'Acad.*, 1902, 135; *ibid.*, 1903, 136.
23. MARTOGGIO E CARPANO. *Ann. d'igiene sper.*, 1904, 14, p. 4.
24. ROSS AND MILNE. *Brit. Med. Jour.*, 1904, 2, p. 1453.
25. KOCH, R. *Deutsche med. Wchnschr.*, 1905, 31, p. 1865.
26. NICOLLE ET COMTE. *Compt. rend. de la Soc. de biol.*, 1905, 59, p. 200.
27. VUILLEMIN. *Münch. med. Wchnschr.*, 1905, 52, p. 1421.
28. CARTER. *Deutsche med. Wchnschr.*, 1879,
29. KARLINSKI. *Fortschr. d. med.*, 1890, 8, p. 161; *ibid.*, 1891, 9, p. 456.
30. MAMUROWSKI. Nothnagel, "Rückfallfieber." *Handb. d. Path. u. Ther.*
31. TICTIN. *Centralbl. f. Bakt.*, 1894, 15, p. 840; *ibid.*, 1897, 21, p. 149.
32. BORREL AND MARCHOUX. *Compt. rend. de la Soc. de biol.*, 1905, 58, p. 362.
33. PASTERNAZKI. See Kolle and Wassermann.
34. KARLINSKI. *Centralbl. f. Bakt.*, 1902, 31, p. 566.

EXPLANATION OF PLATE 7.

FIG. 1.—Usual type of spirochete. Human blood, Case 1. (Wright's stain; $\times 1,500$.)

FIG. 2.—Rat 73. Two short forms, each with three spirals. The lower is greatly thickened. (Wright's stain; $\times 1,500$.)

FIG. 3.—Rat 87. Numerous long forms, joined end to end, or partially approximated. Beginning agglutination (?). In the right upper quadrant two spirochetes are seen, closely opposed throughout the greater portion of their length. (Goldhorn's stain; * $\times 2,000$.)

FIG. 4.—Rat 87. Very long form, consisting of three portions, separated by attenuated intervals. The upper and middle portions are considerably thickened, and ribbon-like. There appears to be a small, unstained cleft in the upper portion. The middle segment shows two deeply staining points, where the flattened spirochete is turned upon itself. (Goldhorn's stain; $\times 2,000$.)

FIG. 5.—Rat 87. Two long spirochetes, end to end, apparently having recently divided. Each spirochete has a thinned-out central portion. Here is a deeply staining granule on one of the organisms. (Goldhorn's stain; $\times 2,000$.)

FIG. 6.—Rat 59. Tube 6. (Citrate normal rat blood; first generation, 48 hours.) Smeared with addition of ascitic fluid. Tangled clump showing tendency to radial arrangement. Moniliform degeneration of individual spirochetes. (Wright's stain; $\times 1,500$.)

* Goldhorn's recent modification of his polychrome methylene blue stain, described at the January meeting of the New York Pathological Society, 1906.



FIG. 1.



FIG. 2.

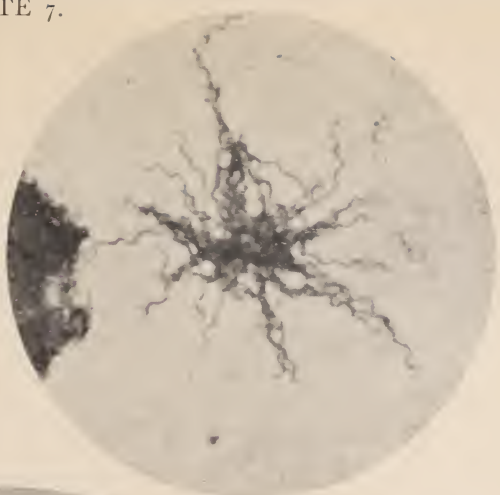


FIG. 6.

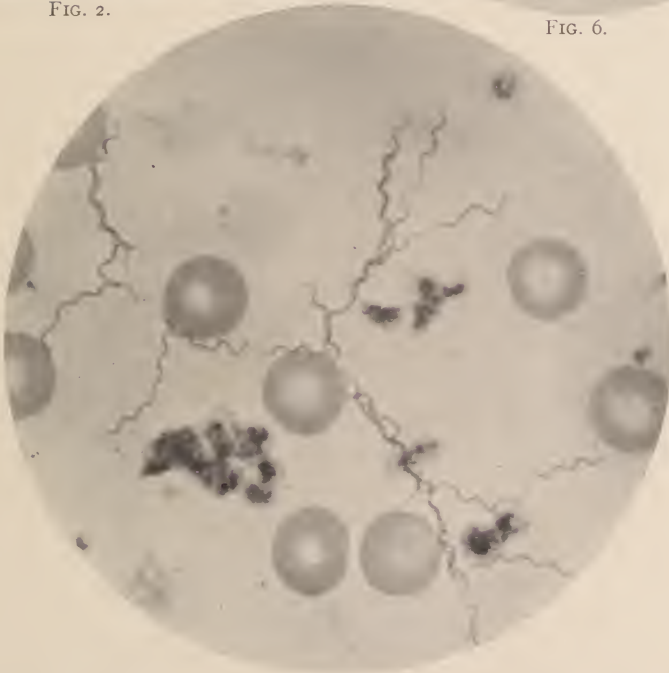


FIG. 3.



FIG. 4.

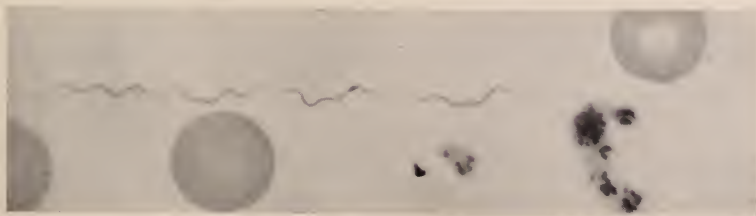


FIG. 5.

STUDIES ON *SPIRILLUM OBERMEIERI* AND RELATED ORGANISMS.*¹

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CONTENTS.

INTRODUCTION.—Summary of spirochetal infections.

NATURE OF THE ORGANISM.—Supposed trypanosome or protozoal character. Evidence against this view based on (1) Structural characteristics; (2) Presence of a flagellum; (3) Transverse division; (4) Rapid multiplication; (5) Plasmodytic changes; (6) Action of heat; (7) Persistence of form; (8) Active immunity; (9) Absence of aërotropism.

MORPHOLOGY.—Short and long forms; two kinds of latter—one due to multiplication, the other to agglutination. Measurements. Flagellum. Character of motion.

VIABILITY.—(1) Extravascular: difference between onset and decline blood; presence of a germicidal body. (2) Intravascular; total destruction of spirochetes *in vivo*.

PATHOGENESIS.—Infection of monkeys, white mice, white rats, wild rats. Relapses in the first two.

ATTEMPTS AT CULTIVATION.—Aërobic and anaërobic; diverse media; pseudo-multiplication; experiments with citrated, uncoagulated blood.

IMMUNITY.—A germicidal substance present in decline and recovered blood; also in hyperimmunized blood. Action *in vitro* and *in vivo*; Pfeiffer's phenomenon; phagocytes ingest dead spirilla. Hyperimmunization: the immunity unit; presence of an immune body. Active immunity: duration; theory of relapse. Passive immunity; duration. Hereditary immunity.

PREVENTION.—Experiments on rats, mice, and monkeys. Great difference in strength of recovered and hyperimmunized blood. Practicability of prevention in man; dose.

CURE.—Absolute cure in rats; relative cure in mice; occurrence of relapses. Cure of monkeys. General considerations as to use of antispirillar serum in treatment of relapsing fever.

AGGLUTINATION.—Action shown by decline, recovered and hyperimmunized blood; *in vitro* and *in vivo*. Identification of spirilla; diagnosis of relapsing fever.

*Received for publication April 17, 1906.

¹A preliminary note on this subject was presented before the Society of American Bacteriologists, at Ann Arbor, December 28, 1905, and was published in the *Jour. Amer. Med. Assoc.*, Jan. 13, 1906, p. 1116; also in *Science*, February 9, 1906, p. 206.

FILTRATION THROUGH BERKEFELD FILTER.—Passage of a filter not a criterion of ultramicroscopic size.

TICK FEVER A DISTINCT DISEASE.—Cause *Sp. Duttoni*, n. sp.; Bombay relapsing fever; existence of a group of relapsing fevers; *Sp. Glossinae*, n. sp., in tsetse flies. Comparative measurements of different spirilla.

SUMMARY.

LITERATURE.

EXPLANATION OF PLATES.

INTRODUCTION.

EVEN before the discovery by Schaudinn and Hoffmann of the *Spirochaeta pallida*, the accepted cause of syphilis, much interest was attached to the spirochetal infections, several of which had been known for a long time. This is particularly true of the spirochete of relapsing fever, which organism was first seen in 1868 by Obermeier, who, however, published no account of his observations until 1873. Notwithstanding that this organism was quite generally recognized as the cause of the disease at an early date, even in pre-bacteriological times, the fact remains that it is one of the least-known parasites. The reason for this is obvious since the disease is exceedingly rare in Europe outside of Russia and the Balkan peninsula. It is apparently common enough in the regions mentioned and also in Asiatic countries, particularly Turkey, Persia, and India. For more than 25 years it has been a rare exotic in western Europe and on this continent it has been practically unknown. Consequently, nearly all of the studies on this disease and its organism have been carried out in Europe by German, and especially Russian workers, and in India by English physicians, notably Carter.

The net result of these investigations was the firm establishment of the relation of *Sp. Obermeieri* to the disease, in spite of the fact that the organism itself resisted all attempts at cultivation. The transmission of the disease to man and monkeys by inoculation with blood containing spirochetes was easily demonstrated, but all other experimental animals withstood such inoculation without any apparent effect. These facts, together with the geographic distribution of the disease readily account for our rather meager knowledge of the organism as compared with that of most of the known pathogenic bacteria. Nevertheless, in spite of these difficulties, attempts were made, as will be seen, to apply a serum therapy and

even a serum diagnosis for the disease. As to the natural mode of transmission of the disease nothing is definitely known, although the bed-bug is commonly credited with being the carrier of the infection. According to several observers, the spirilla remain alive in these insects for an appreciable length of time. Obviously, after having fed on the blood of infected persons these insects must contain in their stomach the parasites together with the ingested blood. Whether actual multiplication takes place in the stomach and whether these insects really transmit the disease is not established. The mere persistence of the organism in such insects for a month or more is a valuable indication which shows that such transmission is possible.

A second spirochetal infection was discovered, in 1890, by Sacharoff, who found it among geese in the Caucasus. The organism known as *Sp. anserinum* is present at times in enormous numbers. It resembles that of relapsing fever, though it is said to be somewhat shorter, and, like the latter, it has resisted all attempts at cultivation. The disease is very fatal, but it is not accompanied by relapses. Death occurs some days after the disappearance of the spirochetes from the blood. It can be transmitted to geese by injecting blood which contains the organism. Ducks and chickens may also be similarly infected, but they recover more easily than do geese. All other animals seem to be immune. The natural mode of infection is unknown, though analogy would indicate insect conveyance.

A third spirochetal disease, likewise very fatal, is that of chickens. This was discovered in Rio Janeiro in 1903 by Marchoux and Salimbeni. The causative organism, *Sp. gallinarum*, is conveyed by the bite of a tick (*Argas miniatus*) when the insect is kept at a temperature of 30°-35°, but not when kept at 15°-18° (Borrel and Marchoux). Chickens have been immunized against the action of the spirochetes, and it has been further shown that the blood of such immune birds possesses marked preventive properties, and Levaditi has been able to demonstrate even a slight curative action. Moreover, in a mixture of immune serum and spirochetal blood, the organisms become immobile and agglutinate, but do not break up into granules or dissolve. The recent demonstration of diffuse flagella and the occurrence of transverse division may be taken to establish the bacterial nature of the organism.

In 1902, Theiler at Pretoria, Transvaal, noted the presence of spirochetes in cattle, and this observation has since been confirmed by Ziemann in Cameroon, and by Koch in East Africa. The infection is very benign and the organism *Sp. Theileri*, is present in but small numbers and soon disappears. It is transmitted through the bite of a tick (*Rhipicephalus decoloratus*), which may transmit at the same time Texas fever. This fact received an admirable demonstration at the hands of Laveran and Vallée, at Paris. They placed some larvæ of this tick, sent to them from S. Africa, on a cow, which as a result, in the course of two or three weeks, developed a double infection of spirochetes and *Piroplasma bigeminum* (Texas fever).

In the same year Theiler also reported the finding of spirochetes in the blood of sheep in Transvaal. Later, in 1904, Martoglio and Carpano also met with this organism in sheep at Erythrea on the Red Sea. It has been assumed, to some extent, that the cattle and sheep spirochetes are the same, though this is not proven. It is advisable that they be considered as distinct organisms until proven otherwise, and from that standpoint we will designate the spirochete of sheep as *Sp. ovis*, n. sp.

Theiler, likewise in 1902, noted the presence of spirochetes in the blood of a horse and very recently (1906) Martin reported another case in a horse at Timbo, French Guinea. Inoculations of sheep and chickens with this spirochetal blood were negative. It is worthy of note that Martin calls attention, the same as Martoglio and Carpano, to a pale central portion indicating transverse division. We propose to designate this organism as *Sp. equi*, n. sp.

Another spirillosis was observed in a bat by Nicolle and Comte in 1905. Its blood on inoculation into two other bats caused infection of both, but was without effect in white mice and a monkey. According to these observers the spirochete also divides by transverse division. We would suggest for this organism the name *Sp. vesper-tilionis*, n. sp.

The spirochete of Vincent which he described in 1894 as occurring with the fusiform bacillus in hospital gangrene, and later in ulcerative angina, affords another example of human spirillosis, though it must be said that the exact rôle played by this organism in these affections is by no means established. The cultivation of this spiro-

chete, under anaërobic conditions and in mixed culture, has been reported by Tunnicliff.

Somewhat similar organisms have been found in recent years, by various observers, in different parts of the body, more especially about the genitals. Thus spirochetes have been found in balanitis, balanoposthitis, condyloma, and smegma; also in ulcerative carcinoma and in tumors of mice. Similar spirochetes have been found in tropical ulcers of man (Patton); also in ulcers of dogs in Delhi (James). Balfour has recently described and figured spirilla in blood clots covering ulcers on dogs and monkeys. Similar, if not identical, spirilla have been known, for a long time, to develop in putrefying blood, and examples of such are given in Plates VI and VII of Fraenkel and Pfeiffer's *Atlas*.

In passing, mention may be made that about 12 years ago one of us met with very actively motile, long spirochetes in a submaxillary abscess in a guinea-pig. They were very abundant and apparently in pure culture, for all cultivation experiments failed to give any growth either of spirilla or of ordinary bacteria.

The constant presence of *Spirochaeta dentium* in the mouth is a well-known fact, and Miller has recently called attention to evidence indicating that this organism possesses pathogenic properties.

In this connection reference should be made to the fact that spirochetes are frequently present, at times in enormous numbers, in the intestines of man. They have been particularly observed in cholera stools (Kowalski (*Spirillum hachaizae*), Abel, Rechtsamer, Lustig and De Giaksa, Escherich, Aufrecht); also in cholera nostras (Grassberger); in diarrhea of infants (Escherich) and even in the dejecta of healthy persons (Paltauf, Escherich). Similar forms have also been observed in the ulcers of the large intestine in a hog (Th. Smith); in a cat (*Vibrio felinus*, Escherich) and in the stomach of dogs, cats, and rats (Bizzozero, Salomon). In all these cases, where attempts at cultivation were made, the result was negative. Escherich, however, reported a successful culture on Naegeli's medium.

Of very great importance is the recognition of the nature of tick fever, a disease which is widely prevalent in many parts of equatorial Africa. Spirochetes were first shown to be present in this

disease in November, 1904, by P. H. Ross and A. D. Milne who made their observations in Uganda. The organism was discovered independently, and about the same time, by Dutton and Todd in Eastern Congo. These workers proved that the disease was transmitted by the bite of a tick (*Ornithodoros moubata*), and they also showed that young ticks, hatched in the laboratory, were able to impart the disease, thus proving the hereditary transmission of the organism from the female to the young. Koch, studying the disease in German East Africa, demonstrated the presence of spirochetes in the eggs as well as in the adult ticks. There is no evidence to show that the organisms multiply in the ticks. The presence of tangles, as observed by Koch, in the eggs of ticks, indicates an agglutinated condition, which, as will be shown later, is far from being a sign of active multiplication. That spirochetes may live without apparent multiplication for 40 days *in vitro* will be shown, and it is probable that a somewhat similar condition obtains in the tick and its eggs. About a fourth to a fifth of the eggs were found by Koch to be infected. Of 645 ticks examined at different places along the caravan road he found 71, or 11 per cent, to be infected. In some places the percentage of infected ticks was small, in others it was large, reaching as high as 50 per cent. It will be shown in this paper that the spirochete of tick fever is distinct from that of the European and Indian relapsing fevers, and in view of this fact we propose to name the organism *Sp. Duttoni* in memory of the brilliant Dutton who lost his life while studying this disease.

The work of Schaudinn and Hoffmann (April, 1905) showed the almost constant presence of spirochetes in syphilis, and their subsequent investigations, together with those of numerous other workers in all parts of the world, have made it more than probable that syphilis is a chronic spirillois. The organism was at first designated as *Spirochaeta pallida*, later as *Spironema pallida*, and still more recently Schaudinn has deemed it necessary to create a new genus of which *Treponema pallidum* becomes the type species.

According to Schaudinn the spirochetes are protozoa and not bacteria. In some, like *Sp. plicatilis* and *Sp. refringens*, he has observed the presence of an undulating membrane but no flagella. In *Trep. pallidum*, however, he demonstrated a flagellum at each

end of the spiral, at times even two, but no undulating membrane. Mention may be made of the fact that Salomon likewise found one whip at each end of the spirillum which occurs in the stomach of cats and dogs. The presence of diffuse flagella on *Sp. gallinarum* and on *Sp. Duttoni* (Zettnow), and of a single whip on the organism studied by us indicates considerable variation among the members of the spirochete group. This matter will be considered more in detail later on.

Bonhoff's observation (1905) of the presence of *Sp. vaccinae* in lymph needs confirmation before any definite conclusion can be drawn. The same may be said of the spirochetes found in yaws by Castellani.

Lastly attention should be called to the presence of true spirochetes in insects. The Sergeants have found such in the digestive tube of a larva of *Anopheles maculipennis*. We have found a similar organism in the stomach of two tsetse flies (*Glossina palpalis*). This will be described later under the heading "*Sp. glossinae*, n. sp." The spirochete reported by Petrie in birds does not belong to this group, but in all probability is a hemogregarine such as we found in sparrows.

The flagellated organism which Schaudinn in 1904 designated as *Sp. Ziemanni* is in reality a trypanosome, and that being the case it should no longer be included in this group. Moreover, in a previous paper we have shown that the supposed relationship of this mosquito flagellate to the intracellular parasite of the owl is open to serious question. Further and convincing evidence on this point has since been obtained from a study of mosquito trypanosomes and of bird hematozoa. The papers on these subjects may be expected at an early date.

The spirochete with which we have been working was derived from the first case of relapsing fever described by Dr. Carlisle in this number of the *Journal*. In view of the clinical features of the disease and the probability of its importation by emigrants from the Far East we have assumed that this organism is identical with *Sp. Obermeieri*. As to whether this assumption is correct remains to be demonstrated by comparisons of living and stained preparations

and above all by animal inoculations and serum tests. There is one noteworthy discrepancy arising from the traditional statement that *Sp. Obermeieri* can be inoculated only into man and monkeys. This is certainly not true, either for the organism with which we have been working, or for that of tick fever, since these can be transferred to rats and mice.

The fact of such transmission to rats, recognized for this organism first by Norris and his co-workers, is of fundamental importance, since by passage through successive animals the organism can be kept alive, and is thus made accessible for prolonged study. In this way, by inoculating one or more rats each day, we have maintained the spirillum since the first week of last November, at which time it was received from Dr. Norris.

NATURE OF THE ORGANISM.

In a paper, published early in 1904, Schaudinn propounded the view that the leucocytozoon of the owl (*H. Ziemanni*), after a process of fertilization in the gut of the mosquito and subsequent asexual division, gave rise to an enormous number of trypanosome-like young forms, which he regarded as spirochetes, and from this he reached the conclusion that spirochetes were flagellate stages of an intracellular organism and as such belonged to the protozoa and not to the bacteria. Hence he designated the large intracellular parasite of the owl as *Sp. Ziemanni*. As will be seen, this designation is a mistake, for, in the first place, the flagellates are not spirochetes; and secondly, they have nothing to do with the intracellular parasite, *H. Ziemanni*.

Of particular importance, in this connection, is his statement that he compared his mosquito flagellates with the *Sp. Obermeieri* and *Sp. anserinum*. As a result of such comparison he arrived at the conclusion that these two forms, in the essentials of their morphology, "Kernverhältnisse, Geisselapparat u. s. w.," agreed completely with *Sp. Ziemanni*. In other words, the spirochetes of relapsing fever and of geese were said to have a definite nucleus, blepharoplast, undulating membrane, and flagellum, exactly as in the case of trypanosomes. Since then, however, Schaudinn has practically withdrawn this comparison, for in a recent publication (October 19,

1905) he states that *Sp. Ziemanni* is *very far removed* from the typical spirochetes, such as *Sp. plicatilis* and *Sp. Obermeieri*.

The view as to the protozoal nature of the spirochetes was quite generally accepted, but recent observations have served to throw serious doubt upon its correctness. Thus, R. Koch in his publication on the spirochete of tick fever has shown that this organism presents evidence of transverse division, and that there is absolutely no evidence of a definite nucleus, blepharoplast, or undulating membrane. At the time flagella could not be detected, but since then Zettnow, employing Borrel's method, has succeeded in demonstrating diffuse flagella. These facts go to show that *Sp. Duttoni* is not related to the trypanosomes but belongs to the bacteria.

Prior to Koch, in fact as early as 1889, Fraenkel and Pfeiffer in their *Atlas* called attention to the transverse division of *Sp. Obermeieri*, which condition is shown clearly in their Plate LXVI. Ten years before this Carter pointed out that the spirillum of Bombay divided into two or three parts. Subsequently the transverse division of *Sp. ovis* was emphasized by Martoglio and Carpano; and more recently a like condition was pointed for the *Sp. equi* by Martin.

Of especial importance is the very recent work of Borrel. He has clearly shown that the *Sp. gallinarum* belongs to the genus *Spirillum* since it divides transversely, is provided with diffuse or peritrichous flagella, and has no undulating membrane. The demonstration of these facts definitely relegates this organism to the group of bacteria. Up to the present, then, two spirochetes, *Sp. Duttoni* and *Sp. gallinarum* have been shown to be non-protozoal in nature. It is more than likely that most if not all of the spirochetes will be returned to their former place among the bacteria.

Our own work, as will be seen, leads to exactly the same conclusion as regards *Sp. Obermeieri*. We have endeavored to approach this question from several standpoints, and all the results obtained point very definitely to the bacterial nature of this spirochete. The evidence on this point will be considered under nine heads.

1. *Structural characteristics*.—The examination of the living preparation failed to reveal the presence of an undulating membrane or of a flagellum, structures which are readily recognizable in even the smallest cultural forms of trypanosomes. The contents of the

cells appeared perfectly homogeneous. The nucleus and blepharoplast, if present, should be easily demonstrable by staining reactions, but such was not the case. The contents, whether stained lightly or deeply by Romanowsky's method, invariably gave a solid stain, exactly as in the case of ordinary spirilla or bacilli. At no time was there the slightest evidence of nucleus, blepharoplast, undulating membrane, or flagellum, all of which, as indicated above, were reported by Schaudinn. Our observations agree with those of Norris and his co-workers, and are in perfect accord with those of Koch on *Sp. Duttoni* and of Borrel on *Sp. gallinarum*. They show conclusively that these spirochetes do not possess the structure of a trypanosome.

2. *Presence of a flagellum*.—Although a flagellum of the protozoal type, easily recognizable either by direct examination of the living preparation or of specimens stained by the Romanowsky method, could not be demonstrated, nevertheless by means of Loeffler's whip stain it was possible to show the presence of a whip having all the characteristics of those seen on bacteria.

From the photographs shown on Plate 10 it will be seen that *Sp. Obermeieri* possesses but a single whip at one end of the short spiral. This is as long as the spiral itself, and the wavy turns of the whip correspond with those of the spirochete. The wavy character of the flagellum should be particularly noted, for, in this respect, it resembles exactly the flagella of bacteria. The flagella of protozoa, such as trypanosomes, are coarse, thick, and do not show any indication of regular wavy bends as is the case with this organism.

The end opposite the flagellum terminates in a short, faintly stained appendage which presumably serves as the starting-point in the development of a new flagellum when division takes place. An examination of the photographs will show that the flagella are present on very short spirals. It would seem as if the longer forms, prior to transverse division, would have a flagellum at each end, but our search for such examples has thus far been unsuccessful. This may be largely due to the method of preparation, as a result of which the longer spirals would probably break up into their smaller components.

At all events it is perfectly certain that the smaller spirals, which probably represent the actual unit or cell, possess but one flagellum

which is terminal. In this respect *Sp. Obermeieri* differs from *Sp. Duttoni* and *Sp. gallinarum* which have been shown to possess diffuse flagella, and from *Sp. pallida* which has a flagellum at each end. The presence of a single flagellum will recall the cholera spirillum, an analogy which, as will be seen later, is borne out to a striking extent in the properties of the immune blood.

The successful staining of the flagellum we owe to Mr. C. T. Burnett, assistant in this laboratory. The method of securing preparations satisfactory for staining purposes, though similar to that employed by Borrel, was devised quite independently. Previous attempts showed the futility of trying to demonstrate the flagella on the spirochetes as they are present in the blood. The excess of organic material was an effectual bar to success, and some method had to be devised whereby clean, washed organisms could be obtained.

It had been noted previously that when rich spirochetal blood was placed in a test tube and allowed to stand for about 24 hours, at room temperature, white patches or islands appeared on the surface of the sedimented corpuscles. These patches were found to be masses of spirochetes which, being lighter, floated above the deposit of red cells. By means of a finely drawn-out pipette these patches were removed with as little serum and corpuscles as possible and transferred to a centrifuge tube. Citrated salt solution was then added to the material and the whole was centrifugated. The clear liquid was then removed, more salt-citrate solution added, and the centrifugation was repeated. Two or three such washings were sufficient to remove the excess of organic matter. The preparation was then mordanted with tannate of iron and stained with a saturated solution of anilin water fuchsin. Best results were obtained by repeated, alternate treatment with mordant and dye. The deposit which formed was cleared up in part by immersion for a few seconds in dilute nitric acid.

3. *Transverse division*.—At the very outset of our work it was noted that the longer spirochetes of the blood when stained showed in the middle a pale transverse band. This pale band corresponds to the faint tips which are seen at each end. This appearance is very suggestive of a cell wall, and as such it indicates that the organism multiplies by transverse division. It is inconsistent with a pro-

tozoal organism which should show longitudinal division. We have looked almost daily for evidence of the latter form of division, in living and in stained preparations, and have never met with the slightest indication of such a change. Moreover, in living preparations we have repeatedly seen a long spirillum separate into two halves. Such observations may be taken as positive evidence of a transverse division, unless it be assumed that in such cases two individuals had become temporarily joined or agglutinated, only to separate later on. End to end agglutination does occur as will be shown later. The union of two cells by means of flagella is well shown in Figs. 4 and 5, Plate 9.

In support of the view that division takes place transversely is the fact that in the earliest stages of infection, before agglutination can be said to occur, the long forms predominate. If longitudinal division was the mode of reproduction one would expect to find at this stage short and thick forms instead of the long ones actually observed.

Our observations on transverse division accord fully with those of Norris and his co-workers. They are also in agreement with those of Borrel on *Sp. gallinarum*; of Koch and Zettnow on the *Sp. Duttoni*; of Martoglio and Carpano on *Sp. ovis*; of Martin on *Sp. equi*; and of Fraenkel and Pfeiffer on *Sp. Obermeieri*.

4. *Rapid multiplication*.—This fact should be taken into consideration in dealing with the nature of the organism in question. At the present time an injection of 0.1 c.c. of spirochetal blood into a rat or mouse is followed by the appearance of the spirochetes in the peripheral blood in about 15 hours. The number then rapidly increases and reaches its maximum in about 36 to 48 hours, after which they decrease and finally disappear. Such rapid multiplication agrees perfectly with a bacterial nature, and is decidedly at variance with all known protozoal infections. In the latter, a period of incubation of several days' duration is the rule and the subsequent multiplication is relatively slow. This is certainly true for the various trypanosomes and plasmodium infections, and something analogous might reasonably be expected if the organism in question was a protozoon.

The fact alluded to above finds a remarkable parallel in the causa-

tive agent of yellow fever, which likewise disappears from the blood in about two days. It goes to show that this unknown organism may be related to the spirochetes, and, as such, may belong, not to the protozoa, as is quite generally accepted, but to the bacteria.

5. *Plasmolytic changes*.—In order to throw further light upon the nature of *Sp. Obermeieri* several series of comparative experiments were made by subjecting the spirochetes, *Tr. Lewisi*, *Tr. Brucei*, the cholera vibrio, *Sp. rubrum*, and the virus of rabies to the action of distilled water. For this purpose the thinnest possible collodium sacs were prepared by the method worked out in this laboratory by Gorsline. These sacs were about 15 mm. in diameter and 60 to 80 mm. long. They were so thin that they collapsed when empty. They were attached to cut test tubes and sterilized at 105° for 15 minutes. The material to be tested was then placed in the sterile sac and suspended in running distilled water. A very good control of the efficiency of the sacs was obtained by placing in one some defibrinated rabbit or rat blood. With a sac of proper thickness such blood will be completely laked in from one to two hours, and a microscopical examination will reveal only the stroma or "shadows" of the corpuscles. Obviously, every sac was subjected to a pressure test to make sure that it was perfectly tight.

When rat blood containing *Tr. Brucei* is placed in such sacs, there is noticed at first a marked hyperactivity of the organism. The motion then becomes sluggish and at the same time the form alters. The posterior end rounds up, and, as a result, nearly all of the trypanosomes assume the kite- or tad-pole-form while the flagella show a sluggish or only occasional motion. Eventually, only round forms can be seen together with tangles of free flagella. In some experiments where the plasmolytic change was very rapid, as shown by the prompt laking of the red blood cells, the trypanosomes were all dead within an hour. This result was usually obtained within two hours, and only exceptionally, when the membrane was not in perfect condition as shown by the persistence of the blood corpuscles, was the *Tr. Brucei* found alive beyond this limit. With one exception mice and rats inoculated with the liquid, dialyzed for two hours, did not become infected.

Similar experiments with *Tr. Lewisi* seemed to indicate a greater

resistance of this organism. The first effect was likewise seen in a pronounced hyperactivity. Within six hours, however, they became swollen and distorted in form and were nearly all dead.

Spirochetal blood, in parallel experiments, under identical conditions, showed an entirely different behavior. The organism became very active and remained so for six to eight hours. They then become somewhat sluggish, and such motion may be observed for 20 hours, although complete hemolysis occurs within a few hours. When the spirochetes come to rest they retain their form perfectly, and at no time is the slightest morphological variation observable.

Furthermore, the spirochetes when they thus come to rest are by no means dead. They may be readily revived, after which they become as active as in the beginning of the experiment. This fact was first observed under the following conditions: A drop of the liquid from a sac was placed under a cover-glass and apparently showed only dead spirochetes. About two hours later the specimen was again examined. In the mean time the liquid had all but evaporated from under the cover-glass. Only a few small patches or ponds were left, and in these we were surprised to find very active spirochetes. Evidently in the evaporation of the liquid the traces of salines, either in the liquid or from the glass, had become concentrated to a point which enabled the vitality of the organism to assert itself. This observation suggested the possibility of restoring the vitality by the direct addition of a salt solution. Accordingly, in one experiment, where the spirochetes, after dialyzing for 20 hours, shows no motion, a drop of the liquid was placed on a slide and to this a drop of a salt-citrate solution (0.5 per cent each) was added. In less than two hours this specimen showed extremely active spirochetes.

These observations show that *Sp. Obermeieri*, unlike *Tr. Brucei* and *Tr. Lewisi*, retains its motion for a longer period, when dialyzed in distilled water; that while the trypanosomes are actually killed under these conditions, the spirochetes merely come to a rest, on account of the decrease in osmotic tension, and can be revived many hours later by the addition of salines.

The above behavior of the spirochetes is similar to that of the cholera vibrio and of *Spirillum rubrum*. For experiments on this

point we are indebted to Mr. R. G. W. Owen, Assistant in the laboratory. The cholera vibrio in a very thin collodium sac in running, distilled water still shows some motion at the end of six hours, occasionally even at the end of 12 hours. The non-motile organism, when transplanted to broth, gives positive growths at the end of 18 hours but not at 24 hours. Suspensions of *Sp. rubrum*, under like conditions, show some motile forms for 12 to 18 and even at times at the end of 24 hours. The non-motile forms invariably give cultures at the end of 24 but not at the end of 30 hours.

It will be seen from the above that the *Sp. Obermeieri*, under the conditions of the experiment, retains its motility as long as, and even longer than, the cholera vibrio and for about the same length of time as the *Sp. rubrum*. In other words, it possesses about the same resistance as these well-known examples of spirilla.

Similar experiments to the above were made with "virus fixe" by Dr. J. G. Cummings, in charge of the Pasteur Institute here. They are of interest inasmuch as they show that the rabic virus is destroyed quite as readily as *Tr. Brucei*—a fact which indicates that the microbe of this disease in all probability belongs to the protozoa. In several experiments the dilute filtered suspension of the virus, after dialyzing for one hour, failed to infect. This result was invariably obtained when the dialysis had proceeded for three hours or more. Controls inoculated with the same suspension, kept under as nearly identical conditions as possible, but not dialyzed, invariably developed rabies. Very thin sacs and very dilute suspensions are necessary here, as in the above experiments, otherwise the virus may not be destroyed completely in from 6 to 12 hours. It is of interest to note that in some of the animals inoculated with the dialyzed liquid a typical excited stage developed, which, however, was of short duration and complete recovery followed. As to whether such recovered animals are immune to a minimum fatal dose remains to be established.

Results somewhat like those with collodium sacs may be obtained by the addition of distilled water to the infected blood. Thus, if one drop of blood containing *Tr. Brucei* is added to 1 c.c. of distilled water the trypanosomes at the end of half an hour will be found to be all rounded up and dead. A similar test with spirochetal blood

will show some actively motile individuals together with many unaltered motionless forms.

The foregoing experiments go to show that dialysis in running distilled water, with the aid of thin collodium sacs, brings out marked differences between the known protozoa and known bacteria. Just how far this difference in behavior will hold true for other representatives of these groups remains to be established by further experiment. It is quite possible that this reaction will be found to be a fairly general means of distinguishing between protozoa and bacteria. In this particular case it certainly proves that *Sp. Obermeieri* differs markedly from trypanosomes, so much so that there can be no question but that it is wholly distinct from the latter. Moreover, the close similarity in the behavior of the spirochete under these conditions to the typical spiral bacteria strengthens the evidence, if it does not actually prove, that this organism belongs among the bacteria and not among the flagellated protozoa.

6. *Action of heat*.—Comparative determinations of the thermal death-point of the spirochete and of *Tr. Lewisi* were made to ascertain if there was any notable difference in this regard between the two types. The defibrinated blood of infected rats was used direct, that is, without dilution. This was taken up in drawn-out, thin-walled tubes about 3 mm. in diameter. The sealed tubes were then immersed in a water-bath, the temperature of which was kept constant at 45°, 50°, and 60°.

In the case of *Tr. Lewisi* an exposure of five minutes at 60° was sufficient to cause the complete destruction, and even the disappearance, of the trypanosomes. After an exposure of five minutes at 50° the trypanosomes were all dead and scarcely recognizable, the bodies being shrunken, rounded up, and granular. In 10 minutes not even dead trypanosomes could be detected. At 45° the trypanosomes were hyperactive at the end of five minutes; at the end of 15 minutes only a few sluggish trypanosomes were present together with many distorted dead forms. At the end of 30 minutes the trypanosomes had entirely disappeared.

The spirochetal blood, drawn during the decline stage, was tested at the same time, side by side with the trypanosome blood. In five minutes at 60° only agglutinated masses of non-motile spirochetes

were present. The same result was obtained in five minutes at 50°. At 45° they were very active at the end of 15 minutes; at 30 minutes there were many sluggish spirochetes present with some small agglutination groups.

According to Heydenreich the *Sp. Obermeieri*, kept at 42.5° to 46°, remains alive for one and three-fourths to three and one-half hours. The difference between his results and ours is easily explainable. In the first place he employed human blood with relatively few spirochetes, whereas we used rat blood which contained about 10 spirals per field of the 2 mm. objective. Of more importance than the mere number of spirochetes is a second factor which is more difficult to control. In the infected animal germicidal substances appear in the blood and their amount increases with the increase in the number of organisms. As will be shown, the disappearance of the spirochetes from the blood of the rat and the absence of a relapse, as in the case of man, is due to the formation of a large amount of such anti-bodies. In our experiments we used a "decline" blood, that is to say, the rats were bled about 48 hours after inoculation, at which time the spirochetes usually reached their maximum number and were even on the decrease. Consequently in such blood the germicidal agent is already present in appreciable amount, and is therefore not without action upon the vitality of the organism. It follows from these considerations that the spirochetes employed in these tests were already in an enfeebled or weakened state. Moreover, it is self-evident that a germicidal agent will act more promptly the higher the temperature. The fairly rapid death of the organism at 45° is largely if not wholly due to the combined action of heat and the specific bactericidal body. It is reasonable to believe that if "onset" blood, in which the germicidal agent is present in minimal amounts, is employed the spirochetes will be found to live considerably longer than 30 minutes at 45°. However, no experiments were made to test this point.

The experiments, such as they are, demonstrate two things: first, that *Tr. Lewisi* at 45° is killed, and disappears in less than 30 minutes; second, that the spirochetes may be alive for more than 30 minutes at that temperature, and that they do not undergo, when dead, any alteration in form. The latter is a particularly important

fact and holds true for all of our experiments, with the exception of Pfeiffer's phenomenon. Even after an exposure of 30 minutes at 60°, the spirochetes, though they agglutinate, show a perfect spiral form, whereas *Tr. Lewisi* disappears almost completely after it has died. The action of the heat, it will be seen, is very much like that observed in the plasmolytic experiments. There, also, the trypanosomes dissolved or disappeared almost completely as soon as they became dead, whereas the spirochetes, though immobilized and eventually killed, retained their form without the slightest alteration.

The absence of any change in form of the dead spirochetes, corresponds to the behavior of bacteria under like conditions and this observation, together with the greater resistance to heat, may be taken as an additional proof of the bacterial nature of the *Sp. Obermeieri*.

7. *The persistence of form.*—In the preceding paragraph special emphasis is placed upon the fact that the spirochetes do not undergo any alteration in form as the result of the action of heat. This fact holds true for all the other conditions which we have studied. Thus, in the dialysis experiments, though the spirochetes became immobilized and eventually dead, yet at no time was there the slightest change in form. The dead cell does not dissolve, or fade away or even become indistinct, but on the contrary it is sharply defined as a rigid spiral. No matter what the condition is which causes the death of the spirochete the latter retains its form perfectly and apparently so for an indefinite length of time. Thus, perfect dead spirals may be seen in blood which has been kept *in vitro* for more than 40 days. In the hundreds of attempts which have been made at cultivation, the dead spirochetes, normal in form, could always be found regardless of the temperature or the medium employed.

Under the influence of the immune serum the spirochetes may be agglutinated and killed in a few minutes, but in such cases also, the typical form persists and not the slightest variation or departure from this can be detected. The only exception to this statement is presented by Pfeiffer's phenomenon, in which, as will be shown later, granulation may result, within or without the body, exactly as in the case of the cholera vibrio.

It will be shown that under the influence of large doses of immune

blood the spirochetes may be killed *in vivo*, and a cure may be sometimes effected within half an hour. In the many tests which we have made along this line we have scarcely ever seen any other than perfectly preserved dead spirochetes. Occasionally, drawn-out or filamentous forms are met with in smear preparations, probably due to traction, since these are not observed in drop examination.

The persistence of the spiral form under the most varied injurious conditions is a fact of much importance, since it is incompatible with a flagellate or protozoal nature. Under adverse conditions protozoa tend to round up, to a greater or less extent, and the absence of any indication of this sort effectually removes this spirochete from the flagellates, and for that matter from the entire group of protozoa.

8. *Active immunity*.—The production of active immunity in several protozoal diseases is well established, though at the same time it must be conceded that immune sera are of little value as curative or preventive agents in such affections. The production of even a relatively active germicidal serum with respect to protozoa is a difficult task which requires much time, usually many months. In the case of the trypanosomes, with the exception of *Tr. Lewisi*, a really active serum is practically unknown.

On the other hand, the bacteria, as a rule, readily react with the production of powerful anti-bodies. Thus, to take one of the earliest and best studied organisms, one which forms a fitting parallel to our spirochete, the cholera vibrio on intraperitoneal injection promptly gives rise to a germicidal agent. The amount of this substance present in the blood of the immunized animal can be rapidly increased by successive injections. In a few weeks it is possible to secure a serum which will act as a preventive or curative agent in extremely small doses.

In the case of *Sp. Obermeieri*, as will be shown later, active immunity can also be produced with an ease which is quite comparable to the example just given. Repeated injections of spirochetal blood cause a rapid increase of the immune and germicidal bodies and to such an extent that the blood of the actively immunized rat has a powerful curative action. Thus, we have been able to obtain a blood which in a dose of 0.002 c.c. protects absolutely a 100 g.

rat against a simultaneous injection of 0.1 c.c. of spirochetal blood. In other words one part of the immune blood protects 50,000 parts of body-weight. The same blood in a dose of 0.5 c.c. causes the spirochetes to disappear, sometimes within half an hour, from the peripheral blood of an infected rat.

The readiness with which active immunity can be established and the ease with which the spirochetal infection can be prevented and cured is to our mind an additional and clinching proof of the bacterial nature of *Sp. Obermeieri*.

9. *Absence of aërotropism*.—During the past three years we have had occasion to study a very large number of cultures of trypanosomes representing many different strains and a considerable number of species. All these cultures present an interesting behavior with reference to air. Thus, if a small bubble of air remains in the liquid under the cover-glass it will be found that the cultural trypanosomes will mass themselves around its border. They form a compact layer of three to five or more cells deep, and each cell points with the flagellum toward the air-bubble. This radial massing about an air bubble appears to be a very general reaction for trypanosomes and as such it may be utilized as a means of separating trypanosomes from accompanying bacteria. Bacteria under similar conditions are in no wise responsive to the presence of air. In some instances it is true that hyperactivity may be observed, but the bacteria never assume the characteristic arrangement as described above for trypanosomes. Furthermore, in our daily examinations of spirochetal blood we have always looked for some evidence of a trypanosome-like behavior with reference to air. This we have never observed. On the contrary, the spirochetes show the same indifference to the air globules as do ordinary bacteria.

This fact is mentioned as an additional proof of the nature of the spirochete.

The several facts which have been brought out under the preceding headings point clearly to the non-protozoal nature of *Sp. Obermeieri*. The only escape from this conclusion is the assumption that there are protozoa wholly unlike those known at the present time. Such an assumption would be unwarranted, to say the least,

especially in view of the marked agreement which the spirochete shows with the well-recognized bacteria. We must conclude, therefore, that the *Sp. Obermeieri* belongs to the bacteria and more especially to the Spirochaceæ. As pointed out, the same conclusion has been reached by Borrel as regards *Sp. gallinarum*, and the facts brought out by him with reference to this organism hold true for *Sp. Duttoni*. That is to say, the latter presents no evidence of nucleus, micro-nucleus, undulating membrane, etc., but does show transverse division and is provided with diffuse flagella. Three typical spirochetes are therefore demonstrated to belong to the group of bacteria. The other spirochetes which show transverse division, as *Sp. ovis*, *Sp. equi*, *Sp. glossinae*, etc., will undoubtedly eventually find their place in the group of spiral bacteria. The wavy flagella on *Treponema pallidum* also indicate a relationship to this group. On the other hand, the position of *Sp. plicatilis* and *Sp. refringens*, which, according to Schaudinn, possess an undulating membrane but no flagella, is less certain. The possibility of capsule formation among the spiral bacteria should not be lost sight of.

As further work is done with these organisms, it may be found necessary to create new genera based upon the number and arrangement of the whips. We now know of spirilla with a bundle of whips at one end, also such as have but one whip, also such as have one at each pole and lastly such as have diffuse flagella. For the present the creation of new genera does not seem advisable and for that reason we will retain the old, established term "spirillum," especially since the type species of the genus Spirochaeta, *Sp. plicatilis* of Ehrenberg, is said to have an undulating membrane but no flagella.

Hitherto it has been assumed that insect transmission indicates a protozoal organism, and in so far as the spirochetes are concerned, the chief evidence which can now be adduced in support of their animal nature is the fact of such transmission in the case of *Sp. Duttoni* and *Sp. gallinarum*. The persistence of the spirochetes in such insects for months, and the infection of their eggs constitute the remaining argument in support of this view. There is as yet absolutely no evidence that the spirochetes actually multiply in these insects, much less any indication of the existence of a life-cycle in any way comparable to that of the malarial organism. The

occurrence of relapses, as will be shown, is readily explainable without the assumption of such a cycle. On the other hand, the facts brought out in this study point so conclusively to the bacterial nature of the organisms that there can be little or no doubt of the correctness of the conclusion arrived at. With the recognition of this proof it follows that insect transmission is no longer a criterion of the nature of an organism.

MORPHOLOGY.

The spiral forms as met with in the blood vary considerably in length, usually from 7 to 19 μ and even more. The short forms vary from 7 to 9 μ in length and hence are as long as, or a trifle longer than, the diameter of the red blood cell. These probably represent the actual size of the young or single cell. The disintegration within phagocytes shows a tendency of the spiral to break up into comma or S forms, and these may represent the actual unit. Further evidence on this point will be brought out under the head of tick fever. The contents of the cell usually stain solid by the Romanowsky method with the exception of the ends, which take a very pale tint and fade away to a point. At times a tendency to granular staining will be observed, but this is probably due to the dye employed. Heavily stained portions or nodes can also be observed, but these cannot be taken as evidence of structure. Examples of the above will be seen in the photographs which accompany this paper.

The long forms may result from either of two wholly distinct causes—multiplication or agglutination. The evidence on this point we consider to be quite conclusive. When the blood is examined at the earliest stage of infection (15 to 18 hours after inoculation) thin, long, and very active spirals can be seen together with the short forms. The presence of these very long cells at the very beginning of infection indicates that they give rise by transverse division to the shorter forms. Their length (16 to 19 μ) corresponds to that of two short forms. An examination of the stained preparation confirms this view in so far as it shows the presence of a pale central zone, corresponding to the pale tips or appendages which can be readily seen on the free ends (see Plate 8).

In the later stages of infection, even longer forms than those men-

tioned appear as a result of the formation of agglutinating bodies. Moreover, when a very active immune blood is injected into an infected rat these very long forms promptly appear in the blood together with agglutination groups of various sizes. The long forms of this type vary in length from about 18 to 100 μ and even more. They result from the end to end agglutination of two or more cells. That such is the case we have been able to determine by the aid of Loeffler's flagellar stain. In a good preparation stained by this method it is easy to find many pairs of cells held together by the flagellum. Two photographs showing this manner of attachment are given in Plate 9. The recognition of this linking by the staining method at once explained a peculiarity frequently observed in fresh blood. It is not an unusual occurrence to find two spirals, separated by an appreciable distance, about the length of the cell, which maintain a perfect alignment and move together, backward and forward. The invisible bond holding together such cells is the flagellum.

The actual width of the spirillum probably does not exceed 0.25 μ . The apparent width depends largely upon the amount of dye which has been deposited in and upon the cell. Hence in feebly stained preparations they may appear as mere lines. The heaviest deposit of dye, as obtained with mordant and anilin water fuchsin, in Loeffler's method, does not increase the width beyond 0.3 μ (see Plate 10). In fresh onset blood the spirilla appear to be thinner than in the latest stages and because of their tenuity and rapid motion they are very difficult to see. In the later stages, and particularly upon the addition of immune blood, the spirochetes become slower and apparently much thicker. They can then be readily seen even by untrained persons.

At times there are found, especially in the later stages of infection, relatively long, thick spirals which are about twice as thick as the ordinary ones. They are usually of the long form and may be considered either as involution forms, or, what is not improbable, as two cells which have become woven together much as in the case of the formation of giant whips. Similar observations have been made by Koch as regards the spirochete of tick fever.

The number of turns in the short spirochetes (7 to 9 μ) which, as indicated above are to be considered as the individual cell, is usually

but two or three. At times the number may be increased to four or even to six which, however, is probably a condition preliminary to division. In the latter kind the turns are but $1.5\ \mu$ apart (from crest to crest) while in the former they are 2 to $2.7\ \mu$ apart. The width of the turns, that is the extreme width of the spiral, is from 0.6 to $1.5\ \mu$, the average being about $1.0\ \mu$. These measurements, it may be added, are best made on photographs of the object which have a magnification of 3,000 diameters; a micron then corresponds to 3 mm. on the photograph.

The short form of *Sp. Obermeieri*, as shown heretofore, is provided with a long flagellum at one end, while the other has a faintly stained appendage, corresponding to the pale tips seen in the ordinary Romanowsky preparation. Tintorially, this behaves like the whip, and may possibly give rise to the latter. The width of the flagellum is probably 0.1 to $0.2\ \mu$, and the length is from 5 to $7\ \mu$. It is usually as long as, or even a trifle longer than, the cell to which it is attached and has from three to five wavy bends or turns. At no time have we seen any indication of the presence of lateral flagella.

The above demonstration of the presence of a single flagellum is at variance with the statement of Karlinski who believed he saw, in living preparations, a pair of whips at each end. No other observer has been able to get any indication of the presence of flagella.

The motility of *Sp. Obermeieri* is exceedingly pronounced, and, as will be shown, it may persist for days and even weeks. Under suitable conditions, such as a proper thickness of the fluid under the cover-glass, the absence of a fibrin network, and of immune bodies, the spirilla will be found to travel with extreme rapidity; so much so that it is quite impossible to follow the organism as it darts in a straight line through the field. This may be regarded as the normal motion of the uninjured or unhindered organism and is particularly in evidence in preparations made during the early stages of infection.

Usually, however, on account of the adverse conditions mentioned, the motion is limited in extent. The cell then travels back and forth over a distance of not more than two or three times its length. It may even adhere, presumably by its whip, to the glass surface or to

the fibrin network, and when this occurs the rapid screw motion continues until the cell is released from its attachment.

The flagellum not only causes the spirillum to move from place to place, but it also effects at the same time a rotation of the cell along its long axis and thus imparts the characteristic screw motion to the organism. Rotation along the long axis is a common occurrence among the rod-shaped bacteria and also among the cultural forms of trypanosomes, and, indeed, must be looked upon as a necessary condition to propulsion, especially where a single lashing whip constitutes the organ of locomotion. Such rotation is easily overlooked in the case of a bacillus, whereas with a spiral organism, on account of its very structure, it becomes a decidedly noticeable feature. Hence, it is manifestly wrong to speak of a real wave motion passing over the spirillum from one end to the other. It is only an apparent motion for the form of the cell is that of a rigid spiral.

The swaying lateral motion, from side to side, is seen only in the long forms which consist of two or more cells. This is also a secondary condition to the real motion, and although it imparts to the long spirochete the so-called flexible character, the latter feature is hardly of sufficient importance to justify its employment as the basis of a generic difference among the spiral organisms. The long form of the *Sp. rubrum* or of the cholera vibrio, as is well known, will show similar lateral swayings.

Under unfavorable conditions the active motion, from place to place, ceases and the organism comes to a rest except for the apparent wave motion which passes alternately backward and forward over the spiral. This motion becomes slower and slower and eventually can be readily followed under the microscope. A brief quiescent stage may intervene before the reverse motion begins. This interval of rest becomes longer and longer, and finally all motion ceases. The cessation of motion does not necessarily imply that death has taken place. Thus, in the dialysis experiments described above, the motionless spirals could be revived under certain conditions. Moreover, we have seen infection result, with the usual period of incubation, by the injection of blood in which all the spirals had lost their motion.

A most important cause affecting the motion and viability of the

spirilla is the presence in the blood of specific germicidal substances. This fact can easily be demonstrated, either by injecting an immune blood into an infected rat, or by drawing the blood during the decline stage and keeping it in a test tube. In either case, the organism soon comes to rest and is apparently dead. As this subject will be discussed farther on it may be dismissed for the present.

The spiral motion is seen at its best in the agglutination rosettes which form *in vivo* or *in vitro* under the influence of immune blood. Hundreds of spirals arrange themselves in perfect radiating rosettes and maintain for hours an extremely active rotation along the long axis. The first effect of the anti-bodies referred to is to induce a marked hyperactivity on the part of the cell. A similar effect is observed when the blood is submitted to dialysis in collodium sacs, as mentioned above.

VIABILITY.

A characteristic feature of infection with *Sp. Obermeieri* is the rapid and even sudden disappearance of the organisms from the circulating blood. In man, monkeys, and mice, after a period of six to seven days, the spirilla reappear and a day or two later they again disappear. Several such relapses may follow each other before final recovery takes place. In the case of a rat there is no relapse, and hence the organisms once gone do not reappear. In view of these facts several interesting questions arise: first, what causes the disappearance of the organisms; second, what becomes of them; and third, how they manage to reappear. The answers involve the question of immunity, and as this subject will be considered by itself, it is undesirable to take up their consideration at this time. Certain facts, however, in connection with the subject of viability have a direct bearing upon the queries propounded.

1. *Extravascular viability*.—When spirillar blood is drawn from a number of rats it will be found that in some cases the organism dies out in about one to two days, while in others they may live for several weeks. We have found living spirilla in blood kept *in vitro* for 40 days, which is by a trifle the longest survival hitherto noted, that of 37 days by Moczutowsky.

The best procedure for obtaining blood from small animals

is to draw it directly from the heart into a sterile pipette. The latter is made from a small test tube the lower end of which is drawn out into a capillary which is then bent at a suitable angle. A narrow glass rod, passing through the cotton plug of the tube, serves to defibrinate the blood. The pipette is sterilized by dry heat. The heart of the etherized animal is exposed, and the cut, flamed tip of the pipette is then inserted. The filling of the pipette is aided by suction, and, when all the blood has been drained, the tip is sealed in a flame after which the blood is defibrinated and transferred to sterile tubes, planted on media, or taken up in a syringe for purpose of inoculation.

The spirillar blood, drawn by the procedure indicated, was transferred in quantities of 1 to 4 c.c. to test tubes, which were then closed with rubber caps and set aside at the ordinary room temperature. From time to time the contents of the tubes were examined microscopically and portions were injected into rats.

In these experiments it was soon found necessary to distinguish between the blood drawn during the early stage of the infection and that drawn during the later stages. The former, for convenience' sake, will be designated as "onset" and the latter as "decline" blood, whereas that drawn after the spirilla had disappeared from the circulation will be known as "recovered" blood.

In decline blood of rats, kept under the conditions mentioned, all the spirilla have been found to be dead in from 36 to 40 hours. This result may even be obtained with blood but 24 hours old. In this time, the corpuscles settle to the bottom, leaving a perfectly clear serum above. Lying just above the corpuscles will be seen several white patches or islands, reference to which has already been made. When these are removed by means of a fine pipette and examined, dense agglutinated masses of thousands of dead spirals will be found. In one experiment two rats inoculated with 0.5 c.c. of such blood failed to develop an infection thus confirming the microscopical observation as to the death of the spirilla. These rats, when reinoculated three days later, each with 0.1 c.c. of fresh spirillar blood, did not become infected, whereas a control which received 0.5 c.c. of the dead spirillar blood plus 0.1 c.c. of fresh spirillar blood became infected. This experiment it will be seen

indicates: first, the presence of a germicidal substance; and second, the presence of an immunizing body. The amount of the latter, however, was not sufficient to protect against a simultaneous injection, rat 2/253, but when given three days before it did manifest a preventive action.

While in the decline blood as shown above the spirilla may die out in from one to two days, an entirely different result is obtained with onset blood. The patches or islands above the corpuscles, if they form at all, are very small and quite numerous. More often they are not formed, but instead the surface of the corpuscles will show a light film which on examination will reveal considerable numbers of very actively motile spirilla, some of which may form small agglutination groups or rosettes. These are insignificant in size compared with the large masses, referred to above, which sometimes fill several fields of the 2 mm. objective. In such onset blood the spirilla will live for a variable length of time. We have found actively motile forms in specimens kept for 30 to 35 to 40 days, and without doubt, with special care, in the selection of the onset blood, they may be found to be viable for a much longer period. It may be added incidentally that under these conditions no evidence of multiplication of the spirilla was obtained.

The presence of living spirilla in kept blood does not necessarily mean that infection of rats can be induced with such material. Thus, a rat which was inoculated with blood 40 days old, in which a few living spirilla were present, failed to become infected. Several days later, it was reinoculated with fresh spirillar blood, but did not develop an infection. From this it will be seen that a small amount of immune body may protect against a very small number of enfeebled spirilla. It should be added that we have infected a rat with blood 37 days old.

It is evident from the foregoing that a germicidal substance, which is not present in blood at the time of the onset, forms as a result of the multiplication of the spirilla. It eventually accumulates in sufficient amount to inhibit their further increase and finally brings about their destruction within the body exactly as it does in the test tube. At times, such action can actually be seen to occur within the body. In the later stages of infection dead spirilla and

even small tangles may be observed; especially is this true when the infection is cut short or modified by an injection of immune blood.

2. *Intravascular viability*.—In the case of animals which suffer relapses it is evident that the destruction of the spirilla is not complete. A small number survive, in some out of the way place in the body, and when the germicidal agent decreases in amount, either by elimination or by oxidation, they reappear again for a short time. Inasmuch as the rat shows no relapse, it seemed particularly well adapted for determining the question as to whether spirilla could live or persist for any length of time in the blood of the recovered animal. It is well known that many protozoa, such as trypanosomes, piroplasmata, and plasmodia, may remain in the blood long after recovery from the disease.

In order to solve this question, three rats were repeatedly examined to determine the exact time of the disappearance of the spirilla from the blood. This was found to be 66 hours in two and 78 hours in the third rat. The rats, 2, 3, 4/232, were then bled 12, 24, and 36 hours respectively after the organisms had disappeared. Two c.c. of the blood of the rat killed after the 12-hour interval was injected into another rat, 3/242. Likewise 1.5 c.c. of the blood of the rat which was bled after 24 hours was injected into a fresh rat, 5/242, and the same dose of the blood drawn at the end of 36 hours was injected into a third rat, 6/242. The rat which received the blood drawn 24 hours after the disappearance developed a mild infection, whereas the other two, inoculated with the 12-hour and 36-hour blood failed to show spirilla. Moreover, these two when reinoculated nine days later with spirillar blood resisted infection.

The variation noted in the above experiment is due either to individual peculiarity of the animal, or, what is even more probable, to the overlooking of a few spirilla. In the latter case the spirilla, instead of having disappeared at the end of 66 hours as was supposed, may have persisted in small numbers for about 80 hours. The detection of a stray spirochete is obviously largely a matter of chance, and hence the supposed interval of 24 hours may have been actually considerably less. This was substantiated by the following check experiment. A rat, 3/77, showed spirilla for about 82 to 84 hours. Its blood, drawn twelve hours later, when injected into a

rat in a dose of 0.5 c.c. not only failed to infect but actually protected rats against a simultaneous injection of spirillar blood.

An objection may be raised to the conclusion that the spirilla are killed off in the recovered blood in 12 hours or less. This may be based upon the immunity result just mentioned and upon a similar result obtained with blood which had been kept *in vitro* for 40 days. In the latter instance, though a few living sluggish spirilla were present, the blood failed to infect and actually conferred immunity. It is therefore supposable that in the negative experiments above the failure to infect was due, not to the absence of spirilla, but to the presence of immune bodies. That such bodies were present was demonstrated by the subsequent failure to infect with spirillar blood. This view, however, has no basis in fact because a germicidal agent is present in such quantities as to destroy rapidly any organisms which might be present. The experiments with decline and recovered blood *in vitro* prove this fact. The recourse to Metchnikoff's theory that the germicidal substance is formed outside of the animal body from damaged leucocytes, is offset by the fact, as will be shown under Pfeiffer's phenomenon, that in actively immunized rats the bactericidal action can be demonstrated within the body.

In his recent paper on African recurrens, Dr. Koch attempts to explain the fact that a large per cent of the ticks containing spirochetes are found in huts in which no cases of the disease exist. One view, which he offers, is that persons who have recovered from tick fever are not free from spirochetes, but on the contrary may harbor a few of the parasites for a greater or less length of time, possibly for years. This assumption is based on the known facts regarding trypanosomatic and piroplasmatic diseases. The close similarity of the *Sp. Duttoni* to *Sp. Obermeieri* leads us to believe that this view is not well founded. In relapsing fever, unlike in the protozoal diseases mentioned, the blood *in vivo* possesses a powerful germicidal and immunizing action and it is inconceivable that the spirilla should exist for any length of time in the presence of such agents. The alternative hypothesis which he gives, namely, that the ticks may derive their spirochetes from infected rats, is more plausible. It is very desirable that an examination be made of a large number of rats in the tick-fever regions to determine whether

these rodents are the carriers of the disease. On the other hand, the viability of the spirochetes in ticks is not fully determined, and it is quite possible, as seen from our experiments *in vitro*, that the organisms may survive for many months in ticks which have fed upon a case of relapsing fever. It would be of interest to know just how long the ticks may retain their infective properties. As a matter of fact, ticks have been sent from Africa to Europe, and, when placed on animals, have caused infection.

PATHOGENESIS.

It is generally stated in literature that *Sp. Obermeieri* can be transferred by inoculation only to man and monkeys. This, apparently, is incorrect as regards mice and rats, which are susceptible to our organism as well as to that of tick fever. The error, if any, of the the early observers may in part be attributed to a failure to examine such animals on the first and second days after inoculation. It is possible, however, that the European organism does not infect these animals in which case it would represent a distinct species. It is therefore very desirable that the spirilla met with in Russian and Indian relapsing fevers be carefully tested as to their behavior in rats and mice. But, whatever may be the cause of this discrepancy, the fact remains that the organism which we provisionally regard as *Sp. Obermeieri* can be transmitted to man, monkeys, mice, and rats.

Rabbits and guinea-pigs appear to be refractory. Thus, two young rabbits, weighing about 800 g. each, were injected intraperitoneally* with 1 c.c. of rich spirillar blood. They were examined daily for 10 days with wholly negative result. Norris, Pappenheimer, and Flournoy have been more successful and have found spirilla in the blood of rabbits on the second day, but none thereafter.

Three young guinea-pigs were inoculated in like manner, and their blood was also examined daily for a period of ten days. Two of these were wholly negative; in the third only two spirilla were found on the third day, but none before or after. It is quite likely that

*To avoid repetition it may be stated that all injections were made intraperitoneally except in the case of monkeys, where subcutaneous inoculation was resorted to.

the stray organisms thus found represent mere survivals of those injected, and that no actual multiplication takes place. According to Sawtschenko spirilla may be found in guinea-pigs for 24 to 36 hours after injection into the peritoneal cavity or into the subcutaneous tissue.

Monkeys.—Inasmuch as Norris, Pappenheimer, and Flournoy have tested very fully the behavior of these animals to the spirillum, it was not deemed necessary to use them except for the purpose of demonstrating the preventive and curative action of immune blood. These experiments will be described in their proper place.

White mice.—The white mice are very susceptible to spirillar infection. The organisms appear in the blood within 24 hours after injection and persist up to about the end of the third day (80 hours). They then disappear for several days, after which a relapse occurs. This is followed, usually, by a third, and even a fourth relapse. The interval between the successive relapses, counting from the appearance of the spirilla in one, to their appearance in the next attack, is about seven days. They may, however, reappear as early as the fourth and as late as the tenth day. So regularly do they return that one can quite accurately predict when this will occur.

The following details serve to show the progress of the infection in mice. Only the days on which spirilla were found are given, the others being negative.

Mouse 6/198, received 0.5 c.c. of spirillar blood on January 13. It was examined daily for 40 days. Spirilla appeared on January 14, 15.

Again	on	January	21	(7-day relapse).
Also	"	"	28	(7 " ").
"	"	February	4, 5	(7 " ").

They were missed on February 11 and although examinations were made until February 22 the spirilla did not return.

Mouse 2/213, received 0.1 c.c. of spirillar blood on January 21. The organisms appeared on January 22. The mouse was then given 0.25 c.c. of an immune blood (result of five injections); the spirilla decreased in number, but did not disappear until after two hours. This was regarded as a cure experiment and no further examinations were made until exactly four weeks later, when it was calculated the parasites should appear, if the cure as not permanent. The examinations made on February 19 and 20 showed a very few spirilla. This represents then a fourth relapse with seven-day intervals (see Monkey No. 1). Examinations made for the next 10 days were negative.

Mouse 6/229 received 0.25 c.c. of spirillar blood on January 29 and its blood was examined every day until February 27. The organism was found on January 30, 31, and February 1;

Again on February 9 (10-day relapse).
And " " 15 (6 " ").

Mouse 7/229 received on January 29 the same injection as the preceding and was subsequently examined for a like number of days. The spirilla were found on January 30, 31.

Again on February 7 (8-day relapse).
Also " " 14 (7 " ").
And " " 21 (7 " ").

Mouse 2/244 received 0.1 c.c. of spirillar blood on February 7. The organisms appeared on the eighth, when it was given an injection of 2 c.c. of immune blood (result of 13 injections). The spirilla disappeared within one hour. No examinations were then made until February 21, after which they were made daily until March 12. Spirilla present February 8, first attack.

Also on February 23.
And " " 27.

The presumption is that a first relapse occurred about February 15, in which case the second and third relapses came on in eight and four days respectively.

Mouse 4/244 served as a control for the preceding. It received the same injection of spirillar blood but none of the immune blood. Daily examinations were made until February 27. Spirilla present on February 8 and 9. Again on February 16 (8-day relapse).

It is evident from the above that relapses occur in treated as well as in untreated mice. The number of relapses varies with different individuals. Particular attention should be called to the fact that the number of spirilla which appear in the blood during a relapse is much less than in the first attack, which clearly indicates the existence of a partial immunity. At first appearance, as many as 50 per field* may appear in the blood, especially is this true when a large dose (0.5 c.c.) is injected. With smaller doses (0.1 to 0.25 c.c.), the number rarely exceeds 10 per field. In a relapse, the number of spirilla is usually quite small; thus, in some instances, but one or two spirilla could be found in a specimen. This was particularly true in the mice which received immune blood. In the untreated mice, the number of spirilla found during a relapse was usually appreciable, from 0.1 to 1 per field.

Recovery in mice seems to be the rule as with other animals. In only one instance have we found what might be interpreted as a fatal result. This mouse, 7/198, received an injection of 0.5 c.c. of spirillar

* The expression "per field" refers to the number of spirilla in a living preparation examined with a 2 mm. objective and No. 1 eyepiece.

blood. On the following, or first day, the organisms were 1 per field; on the second day they were 10 per field; on the third day 30 per field, and the following morning it was found dead. The fatal result may of course, have been due to some other cause than the infection, but the steady increase of spirilla for three days was a very unusual occurrence.

White rats.—The white rat, either pure or piebald, was chiefly employed in this investigation. Up to the present time about 500 of these have been used. No difference was observed in the susceptibility of the two varieties.

As a result of the consecutive passage of the spirilla through this long series an increase in virulence, if this expression can be used, was noted. At first the period of incubation was about 40 hours. The spirilla then became numerous on the second day after the inoculation and even persisted into the third day, after which they usually disappeared. Only very rarely have they been found after 80 hours.

It was soon found that the period of incubation was decreasing and instead of being 40 hours, as at first, it has of late been reduced to 15 to 18 hours. The consequence has been that the rats now show as many as 10 per field, 24 hours after inoculation, and in a few instances it has happened that they disappeared within 48 hours. Usually, however, the spirilla now disappear in about 60 hours after inoculation. Exceptionally, a rat is met with in which the period of infection lasts three or four days. In such which show a delayed infection, the spirilla come on in very small numbers about the third or fourth day and persist for only one day.

Young rats are more susceptible than the old since the spirilla are more numerous and persist longer. They often show as many as 100 per field and in small numbers may be found as late as four and one-half days after inoculation. This behavior of young rats recalls that of children having relapsing fever.

The injection of living spirilla may be said invariably to produce an infection. There are, however, apparently exceptions, for we have met with several rats where living organisms were known to be injected and yet those animals did not become infected. It might be assumed that these few exceptions indicate a natural resistance, and, without doubt, considerable variation due to this factor exists. However,

a more plausible explanation is based upon the fact that immune bodies appear in the blood and reach their maximum about the end of the decline stage. That being the case, it is evident that an injection of only a few living spirilla, and these probably having a lessened vitality, together with a relatively large dose of the immune body, may result in non-infection. An example of this kind has been mentioned in the experiment with blood which had been kept for 40 days. In this case, although a few organisms were present no infection, followed. Several such examples with blood kept in tubes could be given. Similarly, a large injection (0.5 to 1.0 c.c.) of blood drawn at the close of the decline stage may fail to infect. This is particularly true when such blood is extremely rich, 75 to 100 per field, and is kept *in vitro* for a few hours before injection. This explanation, however, does not always hold true. Thus, in one experiment three rats were inoculated, each with 0.25 c.c. of fresh spirillar blood. Two of these developed the usual infection while the third failed.

A number of rats were examined daily for a long period, but at no time could a relapse be observed. In this respect, then, the infection in rats differs from that in man, monkeys, and white mice. It indicates that the cells of the rat readily respond to the presence of the spirilla and give rise to an abundance of immune bodies which persist in the rat and, as a result, all the organisms are destroyed. It will be shown in the part on immunity that rats, which have recovered, retain their resistance for months.

The prompt response of the body by the production of anti-bodies accounts for the remarkable fact that, notwithstanding the severity of infection, death never occurs. In not a single instance among the large number of rats experimented upon has a fatal result been observed.

The only noticeable pathological change in the rat is an enormously enlarged spleen. This was especially the case in the early part of the work when the incubation period was twice as long as at present. Of late, owing to the more rapid onset of the disease, this alteration of the spleen is not as great as formerly, though at times it is still very pronounced.

As to the distribution of the spirilla it may be said that they are present, not only in the circulating blood, but also in all the organs of

the body. This fact has been repeatedly demonstrated on rats from which every drop of blood, so to speak, had been drawn from the heart. The detection of the spirilla in the exsanguinated organs is not easily effected by staining methods. The procedure which we have followed was to add a drop of salt-citrate solution to the moist smear which was then examined for living spirilla. In this way they were detected, without any special difficulty, in the spleen, liver, and bone-marrow.

Wild rats (Mus decumanus).—Several of these, caught in the laboratory, were inoculated with spirillar blood. In one experiment a young rat, 3/20, was given 0.25 c.c. of the infected blood. On the following day it showed spirilla, three per field; on the second day 30 per field; on the third day, three per field, after which they disappeared. In another rat, 6/92, somewhat larger than the preceding, the spirilla did not appear until 72 hours after inoculation. The blood then showed but one per field and on the following day none could be found. The infection in the wild rat is therefore essentially the same as with white rats, although possibly greater individual variation may exist.

ATTEMPTS AT CULTIVATION.

From the very beginning of our efforts to secure artificial cultures of the spirillum it was realized that the problem was an extremely difficult one, perhaps more so than that of the cultivation of trypanosomes. A long series of failures was expected and in this we were not disappointed, for, up to the present time, we have recorded nearly 500 wholly negative results. It is unnecessary to consider these experiments in detail, though a general outline of the ground covered may possibly be of interest.

Several sets of tubes were inoculated each time and these were placed at different temperatures such as 20°, 25°, 30°, 35°, 39°. The tubes kept under aerobic conditions were sealed with rubber caps to prevent evaporation.

A considerable variety of media, chiefly containing blood constituents, was tested. Thus defibrinated rabbit or rat blood or serum was added in different proportions to agar; in other instances the blood or serum was first dialyzed to effect a partial decrease of the saline constituents; and at other times the addition of diverse salts to the

blood or serum was resorted to. In other trials the percentage of the proteid constituents of the blood was decreased and even hemoglobin agar, wholly free from serum proteids, was made use of. Again, the blood or serum was supplemented with auto-sterile brain, liver mush, yolk of eggs, etc.

Anaërobic cultures were attempted by the pyrogallate method with varying partial pressure. Modifications of the glucose-litmus-gelatin process of cultivating anaërobes in the open air, as used in this laboratory for many years, was tried, together with cultivation under neutral oil, with the usual result. The direct inoculation into the yolk of fresh eggs was likewise negative.

Defibrinated spirillar blood, either with or without citrate or glucose solutions, was transferred to tubes and kept under diverse conditions. With these an interesting *pseudo-multiplication* was observed. On standing the corpuscles settle to the bottom, while the specifically lighter spirilla remain in the serum and eventually settle forming a light-colored deposit above the corpuscles. When decline blood is used the spirilla agglutinate and form large patches or islands which lie or float on the surface of the corpuscles. Consequently, when material is removed from this surface layer, it will be found to be enormously rich in very actively motile spirilla, and the first impression is that a successful cultivation has been obtained. A careful examination of the sedimented corpuscles, as well as of the upper layers of the serum, will show an entire absence of the organism. *The apparent multiplication resolves itself into a mere concentration by process of sedimentation.*

Before proceeding to the discussion of another series of experiments, reference should be made to some of the very early investigations which at times are cited as demonstrating extravascular multiplication. The first of these is that of Albrecht (1880), who claimed to have observed an increase in spirilla in blood drawn from the finger before the onset and kept under a cover-glass for some days. Similar observations were made by Lachmann (1880) and by Gerhardt (1881), but these have not been confirmed by subsequent workers. Inasmuch as this work was done in pre-bacteriological times, it is easy to see that contaminations were necessarily present, and hence no value can be attached to such experiments. Reference perhaps should be made

to the statement of F. Cohn (1879) who referred to a letter of Dr. Koch in which the latter claimed to have cultivated the spirillum in the same way as the anthrax bacillus. In view of the fact that no further mention is made by Dr. Koch it may be assumed that he encountered a mere pseudo-multiplication.

The observations of Norris, Pappenheimer, and Flournoy with regard to multiplication of the spirilla in citrated, undefibrinated blood are of fundamental importance, if they can be corroborated. Borrel and Burnet have recently (March 17, 1906) reported like results with *Sp. gallinarum*. In defibrinated blood, with or without addition of citrate solution, we have obtained no evidence of an increase and for that reason it seemed very desirable to repeat the observations of Norris and his co-workers under as nearly the same conditions as possible. We did not, however, employ human blood.

In these experiments the pipette employed for drawing the heart blood was provided with an S-shaped capillary in order to hold back the citrate solution. After sterilization by dry heat, about 3 c.c. of a solution of sodium citrate was introduced into the pipette. Different strength, from 1.5 to 5 per cent, citrate solutions were used. The blood was drawn directly from the heart, through the bent capillary end of the pipette, into the citrate solution, and the two fluids in equal parts were at once mixed by means of the rod, in order to prevent coagulation. Portions of 1 to 2 c.c. of this uncoagulated, citrated blood were then transferred to test tubes which were placed at different temperatures, 25°, 30°, 35°, and 38°. Portions were also transferred to citrated, uncoagulated, normal rat blood.

In these tubes the corpuscles settle in a few hours, leaving above a whitish, cloudy plasma. The latter, on gentle agitation, shows currents of fine particles *strikingly suggestive* of a culture. Moreover, on further standing, a conspicuous whitish deposit, resembling the sediment in fluid cultures of bacteria, formed. The microscopical examination, however, soon dispelled the illusion, for the cloudiness of the plasma and the deposit was found to be due to enormous numbers of well-preserved platelets. The spirilla were in good condition and about twice as numerous as in the original, mixed citrated blood. This increase was apparent and not real, for on thoroughly mixing up the plasma and corpuscles the number was found to be the same as

in the beginning of the experiment. This apparent increase, then, is merely due to the fact that the corpuscles, being heavier, readily settle, leaving the spirilla and platelets in the plasma which makes up about one-half of the total volume.

Cultures were attempted by this method with about 10 rats. The results were no more encouraging than by the other procedures. In a few instances only were scanty, sluggish spirilla found at the end of 48 hours.

In view of the fact that during infection germicidal bodies develop and, in the decline blood, *in vivo* as well as in *in vitro*, actually cause the death or disappearance of the organisms, it is clear that such blood should not be used for culture work. One obvious essential to success, then, is the use of the earliest onset blood (drawn 15 to 20 hours after inoculation), in which the number of spirilla is less than one per field. Such blood, it may be added, was employed in the preceding experiments.

It is worthy of note that in all these cultural experiments the original spiral form of the organisms was found to persist, wholly unchanged, for weeks after they had died. As in the case of bacteria, the dead spirilla retain their form almost indefinitely, and, in this respect, besides many others as shown, they differ from the protozoa.

As to the viability of the spirilla under the conditions tested it should be said that the longest survival is to be noted in plain defibrinated blood, this being in one instance 40 days. On the various agar media, the spirilla may be found alive for two to three days but not longer.

IMMUNITY.

One of the most interesting and, at the same time, most important subjects developed in this study is that of immunity to *Sp. Obermeieri*. The results obtained have been satisfactory in the highest degree, for they not only explain the course of the disease, that is, the crisis and the relapse, but they definitely establish a sound basis for the prevention and cure of relapsing fever and of the related tick fever. Before taking up the discussion of our own work, it may be desirable briefly to indicate the results heretofore obtained by other workers.

In connection with his early studies on immunity, Metchnikoff sought for a germicidal substance in the blood of relapsing fever. He allowed critical or recovered blood, one from which the spirilla had disappeared, to remain in contact with fresh spirillar blood for some hours, and, not observing any injurious action, he concluded a bactericidal agent was absent. The error in this experiment lies in not having employed a highly immune blood, for with such, as will be shown, the destruction of spirilla can be demonstrated within a few hours and even within a few minutes, *in vivo* as well as *in vitro*. In part the error was due, as Gabritschewsky pointed out, to the fact that the test was made at room temperature and not at that of the body. Specific bactericidal bodies may be expected, as in the case of the ordinary enzymes, to act best at, or near, the temperature of the body.

Pfeiffer, in connection with his memorable studies on cholera immunity, postulated the existence in relapsing fever of specific anti-bodies. A short time afterward (1896) Gabritschewsky showed that when equal parts of spirillar blood or serum and normal serum of man or monkeys are brought together, the spirilla will live longer than when recovered blood or serum is used. This difference in behavior was clearly due to the presence of a specific bactericidal body in the recovered blood. In a mixture of spirillar blood and recovered serum, kept at 37°, the organisms died out within less than one hour, whereas when similar mixtures were kept at room temperature a much longer time was necessary. Gabritschewsky accordingly arrived at the conclusion that the cause of the crisis and of the immunity was a germicidal agent.

This view, however, was not accepted by Metchnikoff, chiefly on the ground that Gabritschewsky's experiments were merely reactions *in vitro* and that there was no evidence to show that a similar reaction occurred within the body. He explained the reaction *in vitro* as due to products which escape from leucocytes after these are damaged outside of the body, and not as due to substances existing in the blood at the time it was drawn. Cantacuzène and Bardach have arrived at conclusions supporting that of Metchnikoff. It will be shown in the following that a germicidal substance is formed *within the body* and that its action can be demonstrated *in vivo* either in the circulating blood or in the peritoneal cavity (Pfeiffer's reaction).

Gabritschewsky, as well as previous workers, noted that a monkey which had recovered from the spirillar infection was immune to subsequent inoculation. The latter test was made 21 days after the first inoculation. Active immunity has been found to persist in a monkey for six weeks (Iwanoff), but how much longer this condition will exist is not known. Our experiments on rats show that such immunity may last for at least three months.

In the course of time, with the disappearance of the active immunity, reinfection is possible in man (one to one and one-half years) as well as in monkeys. Such a second attack is invariably mild, is of short duration, and is not followed by relapses.

Passive immunity to *Sp. Obermeieri* was obtained by Iwanoff who injected two monkeys with 20 and 50 c.c. of apyretic blood. In the course of a day or two these were reinoculated with spirillar blood, but no infection followed.

Gabritschewsky was the first to apply serotherapy in relapsing fever, but his results were by no means convincing. In the only experiment made, he inoculated two monkeys with spirillar blood, and on the evening of the third day, 12 hours after the appearance of the spirilla in the blood, he injected into one of them 5 c.c. of the serum from a recovered monkey. The monkey received, 12 hours later, a second injection of 5 c.c. of the serum. Crisis occurred during the following night, and hence the

disease lasted about 48 hours, whereas in the control monkey the crisis occurred after 72 hours. With reference to the monkey which supplied the serum it should be stated that it was first inoculated with several drops of spirillar blood and then 18, 23, and 25 days later it received further subcutaneous injections. Finally, on the 27th day it was given an intravenous injection of 12 to 15 drops of defibrinated spirillar blood. It is obvious from this that the blood which was drawn on the 30th day did not possess a very high immunizing value.

A result similar to that just given was obtained by Bardach, who injected 6 c.c. of serum of a recovered monkey (drawn four hours after crisis) into another one in which the spirilla were just appearing. The spirilla persisted into the evening of the same day, but were gone on the following day. The dose of the recovered blood, however, was insufficient to prevent a relapse on the seventh day.

By inoculating human spirillar blood into three horses Gabritschewsky obtained an anti-spirillar serum which Löwenthal tested on a considerable number of patients. While the latter did not succeed in curing an attack, his results show that a weak serum may be given successfully to prevent a relapse. Of 83 patients thus treated, 39 (=47 per cent) recovered without a relapse, whereas of 140 untreated cases, 65 (=46.5 per cent) recovered after three attacks; practically the same per cent, it will be seen, as of the treated persons who recovered after the first attack.

The results of immunity experiments made with the other pathogenic spirochetes are on similar lines to those indicated above. Thus, Gabritschewsky in 1898 showed that a germicidal substance was formed in the blood of geese inoculated with *Sp. anserinum*, and that its action was specific for this organism; that is to say, the blood of immune geese which killed *Sp. anserinum* in a few minutes at 37° was without effect on *Sp. Obermeieri*, and vice versa; the recovered blood of relapsing fever of man, while it destroyed promptly *Sp. Obermeieri*, was without action on *Sp. anserinum*. The latter lived longest *in vitro* in onset blood. Geese which recover acquire an active immunity corresponding to that of the rat in our experiments. They never suffer relapse and are not reinfected by subsequent inoculation even as late as 113 days. Infection terminates not by crisis, as in relapsing fever, but by lysis, and death usually occurs several days after the disappearance of the spirilla.

He also noted that in serum drawn toward the close of the infection, large agglutination groups formed and that the individual cells rapidly became immobilized and finally dissolved. This he interpreted as a Pfeiffer reaction *in vitro*, the only difference being that Pfeiffer in his cholera work employed hyperimmunized animals. Consequently in the decline stage in geese, germicidal, agglutinating, and bacteriolytic properties were demonstrated. On the other hand, in the blood of recovered geese, while the germicidal action persisted, the bacteriolytic property disappeared, as indicated by the fact that when spirilla were added to such blood they promptly died but were not dissolved.

As in his previous work, Gabritschewsky held that the lysis and recovery of geese was due to bactericidal substances, thus clearly overlooking the presence of specific immune bodies. By injecting intravenously, in four doses, 30 to 40 c.c. of spirillar serum from geese into a horse he was able to obtain a serum which possessed a distinct preventive, but no curative, action. Thus 2 c.c. of this serum injected into geese 24 hours before inoculation with spirillar blood protected, but 1 c.c. failed to do so. The duration of such passive immunity in geese exceeded 20 days, but was less than 30 to 40 days, whereas the active immunity was found to persist for 45 and

even 113 days. Likewise 3 to 6 c.c. of the serum given 24 hours after the inoculation with spirillar blood, and before the organisms appeared in the circulation, afforded protection. On the other hand, 10, 30, and 40 c.c. of the serum had no appreciable effect after the spirilla once appeared in the blood. This result is in agreement with the experience of Löventhal, who was unable to shorten the paroxysms in man with the aid of serum although the latter clearly prevented the occurrence of relapse. It will be seen from our results that the failure to cure with either serum was due to insufficient immunization of the horses.

Chicken which have recovered from infection with *Sp. gallinarum* likewise do not suffer relapses and are not reinfected on subsequent reinoculation. The duration of this active immunity has not been established. Marchoux and Salimbeni induced passive immunity in chicken by injecting blood which contained dead or weakened spirilla. They also showed that the serum of recovered chicken possessed a marked preventive action, either when injected before, or simultaneously with, the spirillar blood. They failed, however, to obtain any curative action; but later Levaditi was able to show that while large doses of immune blood caused death, because of thrombosis due to the agglutination of spirilla, smaller doses brought on a crisis in less time than would ordinarily take place.

In the case of tick fever, Koch has shown that monkeys which recovered from a severe attack were perfectly protected against subsequent inoculation, whereas recovery from a mild or aborted attack did not afford such protection.

THE GERMICIDAL AGENT.

The rat is an admirable reagent for demonstrating the presence of a germicidal body for the reason that its freedom from relapses is probably due to the formation of such substances in larger amount than is the case in man or monkeys. Four methods have been employed to show the presence of a powerful germicidal substance. These, it may be added, have been made use of repeatedly with results so perfectly concordant as to leave no room for doubt that the destruction of the spirilla *in vivo* is accomplished largely, if not wholly, through its action.

The first method consists in comparing the viability of the spirilla in onset and decline blood. As shown heretofore there is an enormous difference in the behavior of the two kinds of blood. Thus in the decline blood which has been kept *in vitro* for 24 to 48 hours no living spirilla may be found, whereas in onset blood they have been observed in living, motile condition for 40 days. The animal experiment confirms the microscopical findings. Thus rats have been successfully infected with onset blood kept for 37 days, whereas decline blood in a day or two loses its infectiveness.

The second procedure is essentially the same except that recovered blood is used; that is to say, blood drawn 12 to 24 hours after the disappearance of the spirilla is used. When such blood is mixed with equal parts of decline blood the spirilla have been observed to die out within an hour at room temperature.

Thus in one test the blood of Rat 3/177 was drawn about 12 hours after the disappearance of the spirilla. Two drops of this were added to a like amount of very rich spirillar blood, at room temperature. In 20 minutes most of the spirilla were dead; a few sluggish cells were found at the end of 25 and 40 minutes. But not a single living organism could be found at the end of 50 and 60 minutes.

The third method consists in the use of blood of an animal which has been hyperimmunized by repeated injection of spirillar blood. One series of experiments of this kind will be sufficient for purpose of demonstration.

Rat 2/127, after receiving 26 injections of spirillar blood in doses of $\frac{1}{4}$ to $\frac{3}{4}$ c.c., was bled and its blood used in the following experiment. It was added to the blood of another rat, which contained about 25 spirilla per field, in varying proportions, 1:1; 1:10; 1:20, 1:50; 1:100.

In the case of the 1:1 mixture, the agglutination took place almost instantly and within two minutes all the spirilla were immobilized.

In the dilution of 1:100, small agglutinations were immediately produced; in 10 minutes the spirilla were very sluggish; in 20 minutes all but a very few were apparently dead. In 35 minutes not a single motile cell could be found.

The dilution of 1:20 behaved in exactly the same manner as the preceding.

The dilution of 1:50, when examined in less than two minutes, showed numerous small agglutination rosettes of 10 to 50 very active spirilla. One or two of these groups were present in nearly every field of the 2 mm. objective. At the end of 30 minutes the agglutination rosettes had broken down to a mass of granules (Pfeiffer's phenomenon), and only a few single, motile spirilla could be found. At the end of one hour the masses of granules had increased, but a very few motile spirilla persisted. The same condition existed at the end of two hours.

In the dilution of 1:100 identically the same results were obtained as in the preceding trial. Some of the agglutinated masses of spirals filled two to three fields of the 2 mm. objective, and, though at first they were made up of actively motile forms, within 30 minutes they had broken down into an unrecognizable mass of granules. Some free actively motile spirilla were present at the end of two hours, perhaps in larger number than in the preceding case.

The fact that in this and the foregoing trials a few organisms survived the intense reaction is explainable on the supposition that

the agglutinating and germicidal substances were insufficient to affect every one of the large number of spirilla present. Exactly analogous results have been observed *in vivo* when the dose of the immune blood employed was not sufficient to destroy, at once, all of the organisms present (see Rat 4/40, below). Moreover, it should be noted that this behavior in a way parallels the lysis observed in geese and the relapses with *Sp. Obermeieri*.

The above experiments clearly demonstrate the presence of agglutinating and germicidal substances in the blood of an hyperimmunized rat when tested *in vitro*.

The fourth method demonstrates that the agglutinating and germicidal bodies exert their action *in vivo*. An indication to this effect is easily observable when the blood of a rat in the decline stage is examined from hour to hour. It will be found that the motion of many of the spirilla becomes quite sluggish; end to end agglutination with the consequent formation of spirals 50 and 70 μ long is quite common, and, at times, even small tangles or agglutination groups may be seen. These, however, are never as perfect and as pronounced as when a large dose of a curative serum is given, as can be seen from the following:

A rat, 4/40, when it had about 40 spirilla per field, was given an injection of 0.25 c.c. of immune blood from Rat 2/127, the same as used above. A drop of blood drawn 30 minutes later and examined at once showed numerous tangles of 10 to 20 spirilla; also long agglutination spirilla, some of which measured 100 μ in length. The rat, re-examined 60 minutes later, showed perfect, radiating, agglutination rosettes composed of very active spirilla. Again re-examined at the end of two hours, the free spirilla were very scarce, not over three per field, and most of these were quite motionless. At the three-hour examination the spirilla were showing signs of recovery from the effects of the injection. They resumed their normal activity, but agglutination groups and spirals, 75 to 100 μ long, still persisted.

In a second experiment 0.5 c.c. of the immune blood, 2/127, was injected into a rat, 6/40, which showed about 20 spirilla per field. In this, as in the preceding, there was a prompt decrease in the number of free spirilla (down to one per field), whereas agglu-

tion groups and long spirals formed. It is hardly necessary to emphasize that, in both these instances, the agglutinating and germicidal actions took place *in vivo*, for the above results were noted on immediate examination of the blood. The motility of the organism was perceptibly decreased, but after three hours recovery seemed to take place.

The above facts which might be multiplied by many similar experiments conclusively prove that a germicidal agent is present in the blood of rats which are in the decline and in the recovered stage and especially in those which are hyperimmunized. Moreover, such blood exerts its specific action *in vivo* as well as *in vitro*. Needless to say that control experiments with normal rat blood show no such action.

Additional examples of germicidal action will be given under agglutination, cure, etc., and especially under Pfeiffer's phenomenon, which will be considered next.

BACTERIOLYSIS AND PFEIFFER'S PHENOMENON.

It has been shown above that under the influence of hyperimmune serum the spirilla, *in vitro* and *in vivo*, agglutinate to form enormous masses and that these *in vitro* soon become an unrecognizable mass of granules. As this reaction corresponds to the well-known Pfeiffer's phenomenon, it was deemed desirable to demonstrate that it takes place in the peritoneal cavity as well as in the test tube. For this purpose a series of experiments were made with: (1) *recovered*, (2) *hyperimmunized*, and (3) *passively immunized* rats. As will be seen these experiments throw a flood of light upon the manner, as well as the rate of destruction, of spirilla within the living body. They effectually do away with the objections which have been heretofore formulated against the humoral destruction of the organisms.

1. *Reaction in recovered rats.*—Rat 5/88, from which the spirilla had disappeared 30 to 36 hours before, was given an injection of 0.5 c.c. of spirillar blood (20 per field). The abdomen was then thoroughly kneaded for about two minutes. At the intervals noted a drop or two of the blood was removed by means of a capillary from the peritoneal cavity and examined direct.

EXPERIMENT A.

Three minutes. Single, hyperactive, distorted or bent spirilla were present in small numbers; apparently instant, positive chemotactic reaction, for the phagocytes, though few in number, are each surrounded by a compact fringe of actively motile spirilla.

Seven minutes. Only three free spirilla found. Phagocytes as before, but the spirilla in the fringe are perceptibly shorter, as if ingestion was slowly taking place.

Ten minutes. No free spirilla found. Phagocytes still fringed with short projecting spirals. Several large masses of granules, probably result of disintegration of spirilla.

Fifteen minutes. Only one sluggish free cell found. Phagocytes have disappeared and reaction is seemingly at an end.

In this experiment the phagocytes apparently ingested still active though enfeebled spirilla.

EXPERIMENT B.

The above rat was given one hour later another injection of 0.5 c.c. of the same spirillar blood. It showed at the end of —

Two minutes. Agglutination groups of 10 to 20 very active cells. One rosette filled half the field of the 2 mm. objective. Only one phagocyte in preparation and this was surrounded by hundreds of spirilla.

Eight minutes. Several, single, dead spirilla; also large agglutination rosettes of very active spirilla. Only two phagocytes found and these were fringed with spirals as above.

Fifteen minutes. One dead and one sluggish free spirillum. Phagocytes as scarce as before. No indication of Pfeiffer's granules.

Twenty-three minutes. Three single and fairly active spirilla and 10 dead; no phagocytes. Reaction almost at end.

It would seem from the foregoing that the first injection had exhausted the phagocytes, which either retired or were killed by the enormous ingestion of spirilla, and hence but very few were available for the second injection. In the absence of phagocytes which exert a marked positive chemotaxis, the spirilla agglutinate about masses of debris, platelets, etc. At the close of the experiment the rat was killed and examined. It showed intensely injected, mesenteric blood vessels.

2. *Reaction in hyperimmunized rats.*—Four rats which previously had received 26 injections of spirillar blood were used for this experiment. Each rat was injected with 0.5 c.c. of spirillar blood (40 per field), after which the examinations were made as above.

EXPERIMENT RAT 3/132.

Two minutes. Showed enormous numbers of phagocytes *but no spirilla, alive or dead*. Masses of granules resulting from the destruction of the spirilla were present.

Five minutes. The same condition as before.

EXPERIMENT B, RAT 4/132.

Two minutes. Enormous numbers of phagocytes; *not the slightest sign of living or dead spirilla.*

EXPERIMENT C, RAT 6/132.

Two minutes. Showed a very few, single, dead spirilla; also a few agglutination groups in one of which a single sluggish spirillum was noted. Enormous numbers of phagocytes some of which show fringes of dead spirilla. The latter retain the spiral form but present a pale appearance.

Preparations, made from this material stained by MacNeal's method, showed the marked phagocytosis as indicated in Plate 11. In some phagocytes the plasma was found to be literally one mass of chromatin granules resulting from the digestion or breaking down of the spirilla. In some cells, comma or S-shaped remnants were still to be made out. This condition can be readily recognized in one or two of the photographs. It is important to note that the *phagocytes are not polynuclear* cells such as Metchnikoff described in the spleen. The study of the origin of these mononuclear phagocytes will be reserved for another time.

EXPERIMENT D, RAT 4/142.

Three minutes later. Showed phagocytes in enormous numbers as before, but no sign of dead or living spirilla.

The above experiments show that the destruction of spirilla in the peritoneal cavity of hyperimmunized rats is almost instantaneous. In only one out of four experiments could the spirilla be detected two minutes after injection. The phagocytes are all mononuclear cells, and, under the conditions of the experiment, it must be assumed that they ingest spirilla which were previously killed by the germicidal constituent of the body fluids. It is further noteworthy that the intracellular digestion of the spirilla takes place with enormous rapidity. The almost instantaneous destruction and digestion of the countless spirilla introduced can be well compared to the rapidity of a chemical reaction. These observations are of interest as bearing upon the rate of the metabolic processes in the animal body.

3. *Passively immunized rat, 1/89.*—This was given an injection of 0.5 c.c. of blood from the hyperimmunized Rat 6/132 used in the above experiment. It was reinoculated 24 hours later with 0.5 c.c. of spirillar blood (50 per field). The fluid was drawn from the peritoneal cavity and examined as before. It showed at the end of—

Two minutes. An intense positive chemotaxis. The phagocytes were very numerous and every one was fringed with enormous numbers of very active spirilla. Intense hyperactivity is a feature. Some free agglutination rosettes, also dead, single spirilla were present.

Six minutes. Exceedingly active, single spirilla are fairly common. Some dead

spirilla also met with; also loose tangles and perfect rosettes. Phagocytes fringed as before.

Twelve minutes. Condition about the same as above.

Twenty minutes. Condition unchanged.

Thirty minutes. The most notable feature is the great and sudden decrease in the number of phagocytes. The excessive hyperactivity continues. A few single spirilla are present, also many long forms 50 to 100 μ ; also rosettes of 20 to 100 very active spirilla.

Forty minutes. Phagocytes are very rare and are fringed with active spirilla as in the beginning. Rosettes are very numerous and are composed of very active cells.

Fifty minutes. Each field has two to three rosettes consisting of several hundred very active spirilla. A few fringed phagocytes persist as before. The free, single, actively motile cells are apparently more numerous; no sign of dead cells.

Apparently the spirilla are recovering from the effects of the agglutinating and germicidal agents.

Sixty minutes. Rosettes are not as numerous, are smaller and less compact than before. Few phagocytes, fringed with active spirilla, still present. *Actual increase* in number of free, single, actively motile forms.

In the above experiment, the spirilla seemingly recovered from the effects of the anti-bodies in about one hour. The change was so marked that a general blood infection was confidently expected on the following day. This, however, did not occur, and at no time during the subsequent daily examinations were spirilla found in the blood of this rat. The passive immunity was therefore sufficient to protect against the enormous dose of spirilla, although, at the end of the first hour the indications pointed to a probable infection. It would seem as if the immediate resources of the passively immunized rat were insufficient in the first struggle, but that reinforced later by additional newly made anti-bodies they eventually destroyed the organisms before general infection occurred.

The significant feature of this experiment is the inability of the phagocytes to destroy the living spirilla. The rather sudden disappearance of most of the phagocytes in about 30 minutes corresponds to the observations made above with recovered rats. On comparison it will be seen that the reaction in the recovered rat is much more intense than in one passively immunized in the manner indicated.

From the three series of experiments outlined above it will be seen that Pfeiffer's reaction is almost instantaneous when a hyper-immunized rat is employed. The germicidal and bacteriolytic actions go hand in hand and the phagocytic ingestion is probably

consequent upon the death of the spirilla. The reaction as observed in recovered rats is less rapid, but nevertheless clean cut. The germicidal and bacteriolytic actions are present as before but in less degree, and there is an apparent phagocytic destruction of living though weakened spirilla. The reaction in the passively immunized rat was the least marked of the three, though the same defensive properties were brought into play.

PRESENCE OF AN IMMUNE BODY.

It has been shown conclusively, in the preceding part, that germicidal and agglutinating substances are present in the blood as the result of spirillar infection. It becomes of interest to establish the similar presence of an immune body. The latter is not necessarily identical with the germicidal substance; indeed, there is good reason to believe that they are wholly distinct. Thus, it can be shown that blood which has little, even minimal, germicidal action is yet capable of preventing infection. As pointed out heretofore, Gabritschewsky considered immunity to be due to the presence of a specific germicidal agent. One of his own experiments clearly shows that this is not the only factor. Thus, the bactericidal coefficient of the serum of an immune monkey was but a trifle above that of a normal serum, and yet, notwithstanding this evidence, he concluded that protection was obtained even when this coefficient was comparatively low.

The evidence as to the presence of an immune body in the blood, during and after infection, will be presented under the following heads:

1. *Inoculation with old kept blood.*—In defibrinated blood, which is kept in capped sterile tubes for a varying length of time, the spirilla die out, soon or late. It was repeatedly observed that when such blood was injected into a rat, even if it contained a few living, but sluggish spirilla, no infection resulted. The inoculation of such rats a few days later with spirillar blood invariably gave negative results. Such tests clearly show that the germicidal constituent must be very small in amount, if any, and consequently, the protection must be due to some other agent. In other words, an immune

body is present in the kept blood and persists long after the spirilla have died out. The following examples are selected from many to illustrate this point.

Rat 5/10 received on February 22 an injection of 0.5 c.c. of spirillar blood, 6/183, kept at room temperature for 46 days. Daily examinations of the rat were negative. It was reinoculated on February 26 with 0.1 c.c. of fresh spirillar blood. Result equally negative; control positive.

Rat 6/10 inoculated on the same day as preceding with spirillar blood, 4/191, kept 40 days under like conditions. Daily examinations were negative. Reinoculated on February 26, with 0.1 c.c. of fresh spirillar blood, was found to be immune.

Rat 7/10 inoculated at the same time as the preceding with spirillar blood, 1/198, kept 38 days as above, failed to develop infection and when reinoculated on February 26 with 0.1 c.c. of fresh spirillar blood gave like result, showing that immunity had been conferred by the previous inoculation.

Experiments like the preceding show conclusively that immunity can be obtained by means of a blood which does not contain a sufficient amount of the germicidal substance to destroy the spirilla until after the lapse of a month or more. In other words, they show that the germicidal and immune bodies are distinct.

2. *Inoculation with blood one to two days old.*—The presence of a germicidal substance in decline blood has been demonstrated above, and, as pointed out, in such blood when drawn near the close of the infection, the spirilla may die out within 24 to 48 hours. No living organism can be found at the end of that time in the blood, and injection of such into a rat will fail to produce infection. The rats thus treated on subsequent injection with fresh spirillar blood will not become infected. This fact, like the preceding, points to the presence of an immune body in the drawn blood. However, the non-identity of the latter with the germicidal agent is not as clear as in the first set of experiments.

Rat 1/10 was inoculated on February 21 with 0.25 c.c. of spirillar blood, 1/7, which had stood for 24 hours in the room. No infection resulted and the rat was found to be immune to subsequent inoculation with fresh spirillar blood.

Two other experiments of the same kind are referred to under the head of "Extravascular Viability." It will be noticed from the control mentioned (2/253) that 0.5 c.c. of this dead spirillar blood immunizes when given three days before, but not when given simultaneously with 0.1 c.c. of the spirillar blood.

3. *Inoculation with recovered blood.*—The disappearance of the spirilla from the blood is largely, if not wholly, due to the action of the specific germicidal substances. And, in view of the preceding experiments, it is evident that such recovered blood must be capable of conferring immunity, though naturally enough it leaves the question open as to whether such immunity is due to the germicidal substance or to an immune body. One experiment of this kind (see "Intravascular Viability") shows clearly the immunizing properties of the recovered blood. Such blood, however, is rich in the germicidal body as seen from the fact that when mixed with an equal part of spirillar blood it will destroy all the organisms in less than one hour.

Another very instructive experiment is presented in the following: A rat, 3/77, was bled 12 hours after the disappearance of the spirilla from its circulation. The given dose of this blood was mixed with 0.1 c.c. of fresh spirillar blood and injected at once into a rat. The several tests are given below in a tabular form.

RAT, No.	SIMULTANEOUS INJECTION OF		RESULT
	Recovered Blood	Spirillar Blood	
3/88	1.5 c.c.	0.1 c.c.	No infection
4/88	0.75	0.1	" "
5/87	0.5	0.0	No infection and when reinoculated 4 days later with 0.1 c.c. spirillar blood it became infected.
			See below
9/90	0.5	0.1	No infection
10/90	0.5	0.1	" "
3/92	0.1	0.1	Very slight infection 3 days later
4/92	0.2	0.1	" " " 4 " "

It will be seen from the above that while 0.5 c.c. of the recovered blood protects against a simultaneous injection of spirillar blood it does not show this action when given four days previous to the latter.

The injection of an emulsion of half the spleen of Rat 3/77 failed to infect Rat 6/87. This rat, when reinoculated with 0.1 c.c. of fresh spirillar blood at the same time as Rat 5/87, developed a very slight infection on only the fourth day. The long period of incubation and the mild infection is in striking contrast with Rat 5/87, in which the infection was prompt, the spirilla appearing on the

first day and persisting for three days after inoculation. It would seem from this single trial as if the spleen was richer in the immune body than the blood. In another experiment, somewhat similar to this, a rat was bled shortly after the disappearance of the spirilla from its blood. Its blood in a dose of 1 c.c. produced infection in a rat, whereas a thick emulsion of the spleen failed to infect and actually imparted immunity.

4. *Inoculation with filtered blood.*—The details illustrative of this point will be discussed later under a separate head. In this connection, however, it will be sufficient to make the general statement that filtered blood when it does not actually cause infection may confer partial or complete immunity. That is to say, rats which received 5 to 10 c.c. of a Berkefeld filtrate of diluted spirillar blood, without becoming infected, when inoculated several days later with fresh spirillar blood, developed either a very slight and late infection, or none at all. This evidence is essentially the same as that presented under 1. In both these instances, blood was employed before the decline stage had set in, and consequently it was relatively weak in germicidal bodies. In the one set of experiments the organisms were allowed to die off slowly in about five or six weeks, whereas in these trials they were removed by the filter.

5. *Inoculation with dialyzed blood.*—It has been shown heretofore that, when spirillar blood is dialyzed in running distilled water, the spirilla retain their vitality for a much longer period than do the trypanosomes. In one experiment, after dialyzing for 20 hours, 2.5 c.c. of the laked blood, containing apparently dead spirilla, were injected into Rat 2/251. The subsequent daily examinations being negative the rat was reinoculated four days later with 0.1 c.c. of spirillar blood with like negative result. Controls positive. In the dialyzed blood, the spirilla were not actually dead, for it was possible to resuscitate them under a cover-glass, and yet they failed to cause infection as did also the subsequent inoculation with fresh active spirilla. This result, together with the preceding, is clearly due to the presence in the blood of immune bodies which appear early, even before the decline stage.

Inoculation with heated spirillar blood can also be resorted to in order to demonstrate the presence of an immune body; a proced-

ure which Marchoux and Salimbeni have followed with *Sp. gallinarum*.

6. *Inoculation with hyperimmunized blood*.—This affords final and positive proof, if any be needed, of the presence of an immune body. As will be shown, the blood of a rat which has been actively immunized possesses a very marked preventive and curative action, sufficiently so to justify the belief that when the artificial culture of the spirilla is once realized, the sero-therapy of relapsing fever, as well as of tick fever, will be an accomplished fact.

ACTIVE IMMUNITY.

The fact that man is immune to subsequent infection, after recovery from relapsing fever and from tick fever, is abundantly demonstrated by clinical observations. Moreover, the experimental confirmation of this fact has been supplied by many investigators, and reference to some of these observations has already been given.

The duration of this active immunity has not been fully established, but in some experiments on monkeys it has been found to persist for six weeks (Iwanoff). There is reason to believe that it will last much longer. Norris, Pappenheimer, and Flournoy have shown that monkeys inoculated with their spirillum likewise became immune to subsequent inoculation.

In our own work on rats and mice we have demonstrated by numerous trials that these animals, when once recovered, become wholly immune. This condition can be easily demonstrated in the case of rats. As shown heretofore, on inoculation with spirillar blood the organisms appear in the blood in from 15 to 40 hours, depending upon the number of successive passages, they then persist for 24 to 48 hours, rarely for a longer time, after which they disappear for good. No relapse has as yet been observed in a rat. That the rat from whose blood the spirilla have disappeared is immune is shown by the fact that on subsequent inoculation no infection results.

The duration of the active immunity, thus established, is of interest. The exact length of time which elapses before the rat becomes again susceptible has not been determined, but that the immunity conferred is solid and lasting, of that there can be no doubt. We have repeatedly endeavored to inoculate rats one, two, and

three weeks after a previous infection with wholly negative results. The following experiments will clearly show that the immunity is not of short duration.

Two rats, 7/114 and 8/114, which were inoculated with spirillar blood on November 27, were reinoculated 55 days later, on January 21, with 0.25 c.c. of spirillar blood (10 per field). No infection resulted.

Rat 2/116 received its first inoculation on November 28. The spirilla persisted until December 1 and were gone on the following day. Reinoculation was made on January 21 (54 days later) with negative result. Controls positive.

Rat 1/131 was inoculated with 5 c.c. of a Berkefeld filtrate on December 7. The number of organisms in the filtrate was evidently very small, for they did not appear in the blood until December 10 and were gone on the following day. This rat was reinoculated on February 11 (66 days later) and failed to become infected, whereas the control was positive.

Rats 3, 4, 5/138 were inoculated on December 9 with 5, 5, and 7 c.c. respectively of a Berkefeld filtrate. All three became infected. They were reinoculated on March 27 (108 days after the first inoculation), but did not become infected. Control positive as usual.

In white mice, as in rats, a condition of active immunity is established but with this difference, that a solid, lasting immunity is not acquired until after one or more relapses have taken place. That a condition of active immunity exists in a mouse at the close of the first attack can be readily demonstrated. Thus, if a mouse from whose blood the spirilla have disappeared, a few hours before, is given an injection of 0.25 c.c. of richest spirilla blood (50 to 100 per field), it will not become infected but a relapse may follow in the usual time.

The theory of relapses.—The condition at the close of the first attack in a mouse, monkey, or man may be designated as a relative or temporary immunity which is due, in the first place, to the destruction of the spirilla by the specific germicidal agent, and secondly, to the presence of a specific immune body. The amount of the germicidal substance is sufficient to kill or weaken most of the spirilla *which are then taken up* by the phagocytes. Some of the spirilla, however, escape this fate because they are sheltered in some part of the body, possibly in extravascular places. Eventually, as the result of a partial elimination or oxidation, or both, of the anti-bodies mentioned, the spirilla are enabled to multiply and hence reappear in the blood. In the resulting relapse, which occurs usually after an interval of about seven days, they are never as numerous as at first and, as a rule, in each suc-

cessive relapse they appear in less numbers. Each such relapse has the same effect as an injection of spirillar blood, and hence after several such attacks a condition of solid immunity is established, due chiefly to the presence of a sufficient amount of immune bodies or to an increased production of such substances.

It is our belief, based upon experimental evidence, that the germicidal and immune bodies are two distinct substances and that the relative amounts of these two substances at the close of the attack determine whether or not a relapse will occur. It is wholly unnecessary to assume the existence of spores, as many of the older writers have done in order to explain a relapse. Neither is it necessary to assume that the organism passes through a cycle analogous to that of the malarial parasite. The fact that the spirillum is not a protozoon effectually eliminates this theory of a cycle. The survival of a few normal spirilla in some protected spot in the body is quite sufficient for this purpose. Moreover, that such a survival is possible is indicated by the observation of Cantacuzène, who, after the complete disappearance of the spirilla from the blood, found them present in the vascular papillæ of feathers and even in the pericardial exudate of geese, previously infected with *Sp. anserinum*.

The view expressed above is based upon a large number of observations made on rats and mice. When these receive injections of blood containing very small amounts of immune bodies the spirilla are retarded in their appearance and eventually are found in very small numbers, perhaps but one or two on a cover-glass. Rat 1/131, referred to above, will serve as an illustration. In that experiment the filtrate injected, as will be shown later, contained immune bodies as well as a small number of spirilla, and the result was a delayed and very mild infection, which, in spite of this fact, conferred an immunity lasting more than 66 days. The mild infection in such cases is comparable to the relapses mentioned above.

In view of the fact that mice, as well as man and monkeys, are subject to relapses, it might be expected that, after complete recovery has taken place, the immune bodies would be eliminated or oxidized more readily than in the rat, and hence the duration of such immunity would be shorter. To test this point a number of mice which had apparently recovered were injected with fresh, rich, spirillar blood on March 12, with negative result. The data are as follows:

Mouse 6/229, inoculated January 28; the second and last relapse occurred on February 15. Interval before reinoculation, 25 days.

Mouse, 7/229, inoculated also on January 28; the third and last relapse occurred on February 21; interval before reinoculation, 19 days.

Mouse 4/244, inoculated on February 7; the first and last relapse occurred on February 16. Interval before reinoculation 24 days.

The failure to infect these mice shows that the duration of the active immunity in these animals exceeds 25 days. Whether it is actually shorter than that of the rat we are not able to state, but in view of the rather long duration of passive immunity in mice it would seem that the active immunity would last for several months.

HYPERIMMUNITY.

This condition can be readily established in rats by repeated injections of spirillar blood. In our series about 30 rats have been thus immunized. The spirillar blood, drawn about 45 hours after inoculation and containing 10 to 50 spirilla per field, was injected in doses of 0.25 to 1.0 c.c., intraperitoneally, every other day. The majority of our rats have received from 26 to 32 of such injections, and hence have been under treatment for over two months. They tolerate these inoculations perfectly, and, so far as the danger from intraperitoneal injection is concerned, that is practically *nil*. Among the hundreds of rats and mice which we have injected in the course of this investigation, probably not more than one or two died as the result of this method of inoculation.

The repeated injection of spirillar blood results not only in an increase in the amount of agglutinating, germicidal, and immune bodies, but also in the production of precipitins or anti-complements, and possibly even anti-immune bodies. The so-called anti-complement action was particularly noted in the blood of rats which had received the maximum number of injections. As the effect of such action is to inhibit or lessen the preventive and curative power of a serum it obviously must be taken into consideration. The following will serve to illustrate this apparent variation in the strength of an immune blood.

Rat 5/145, fairly rich in spirilla, was given an injection of 1 c.c. of an immune blood, obtained after eight injections. Within five hours

the spirilla completely disappeared. A number of similar experiments with immune blood resulting from seven, eight, and thirteen injections caused the disappearance of the spirilla in from one to two or three hours. When therefore a rat, 2/127, which had received 26 injections of spirillar blood was bled, it was expected that its blood would accomplish the same result in a dose of 0.5 c.c. or even less.

Accordingly two rats, 4, 6/40, rich in spirilla, received 48 hours after inoculation an injection of 0.25 and 0.5 c.c. respectively of the blood of Rat 2/127. It was a matter of surprise to find that these injections had only a very slight effect on the spirilla, which persisted for more than 72 hours after the first inoculation. This surprising result was explained by assuming the presence of an anti-complement action. To determine this point, the above experiment was repeated with this difference, that the dose of 0.5 c.c. of the immune blood was added to about 5 c.c. of normal defibrinated rat blood and the mixture was set aside at 36° for about one hour.

Two rats, 2, 3/55, in the onset stage and containing spirilla about one per field, received each an injection of the above mixture, kept at 36°. From the second, the spirilla disappeared within one hour, and from the first within two hours. The only difference between this and the preceding experiment was, first, that the immune blood was injected in the former, mixed with salt solution, while in the latter, it was mixed with normal rat blood and digested at 36°, for one hour in order to neutralize the effect of the anti-complement or precipitin bodies; and second, that the rats in the former set were entering on the decline stage, while in the latter they were still in the onset stage. The cure in the onset stage is probably even a more severe test than if it had been attempted in the decline stage. This experiment may therefore be considered as demonstrating the presence of "anti-complements," the action of which can be neutralized by allowing the immune blood to stand with an excess of normal blood for one to two hours at 36°.

As a result of these observations the immune blood in most of the subsequent work was treated in the manner indicated. Normal blood has little or no action on the organisms, and hence can be injected in large amount without influencing the course of the infection.

For the purpose of comparison it is desirable to establish a standard

or unit of strength for the immune blood. The *unit* which we employ may be defined as that amount of immune blood or serum which on simultaneous, intraperitoneal injection with 0.1 c.c. of rich spirillar blood will just protect a 100 g. rat against infection. The strength is then expressed as so many units per cubic centimeter. The spirillar blood is always drawn about 45 hours after inoculation, and contains from 10 to 50 spirilla per field. The dilution of the immune blood may be made with salt solution, but it is better to dilute with normal rat blood allowing the mixture to stand at 36° for one hour. Obviously such a standard is not absolutely accurate, but it is sufficiently so for all practical purposes.

In applying this test it is important to remember that the immune blood possesses a powerful germicidal action, and, consequently, if the mixture of immune and spirillar blood is allowed to stand for any length of time, the organisms may be weakened or even killed. Any injurious action *in vitro* of this kind would necessarily give false values. To obviate this effect, we have always diluted the immune blood with 2 to 3 c.c. of salt solution before adding the spirillar blood. The resulting dilution was at once injected into the test animal. It may be advisable, in order to counteract this injurious action, which is more noticeable the stronger the immunity, to resort to separate injections of the immune and spirillar bloods in different parts of the body.

According to the definition given, if 0.5 c.c. of recovered blood, 3/77, protects, in the manner mentioned, it follows that it contains in 1 c.c. two immunity units. A hyperimmunized blood, such as that of Rat 2/127 used above, protects in a dose of 0.002 c.c. and hence it contains 500 units per cubic centimeter.

The example just given shows the comparative strengths of recovered and hyperimmunized bloods. It will be seen that while 1 c.c. of recovered blood has but about two units, that of a hyperimmunized rat is 250 times as active. It is safe to say that this by no means represents the maximum strength attainable. We hope to be able to have a blood which will contain twice as many units as that given above, and, if cultures of the organism were at one's disposal, there should be no difficulty in securing a blood having 100 or 1,000 times the strength of that used above. It should be noted that our figures refer to de-

fibrinated blood and not to serum. We have employed the former almost exclusively as a matter of economy in time and material. Obviously the strength of the respective sera would have been about twice that given above.

PASSIVE IMMUNITY.

This condition can be readily established in rats, mice, and monkeys. The methods of demonstrating the presence of an immune body, discussed above, may also serve to illustrate the production of passive immunity. Inasmuch as it is the basis of the important problem of prevention and cure of relapsing fever, further examples will be given under these heads.

Passive immunity, it will be seen, can be obtained by the injection of filtered, or dialyzed (possibly heated), blood; also, with such in which the spirilla have died out or become enfeebled. It may be objected that the immunity established by either of these procedures is an active one due to chemical products of the organism. This, however, hardly seems to be a correct explanation, for everything points to the presence of an immune body from the very onset of the infection. Hence, the immunity resulting from the use of such blood is in all probability a borrowed one. This is particularly seen when simultaneous injections of such blood and spirilla are made, for the immediate protection obtained under such circumstances can only be explained by the presence of an immune body.

Rats and mice can be passively immunized with ease by means of a hyperimmune blood. Much less than one unit will suffice for this purpose provided the immune blood is injected one or two days in advance of the spirillar blood. On the other hand, one unit may fail to protect if given four days previous to the injection of the spirilla (see p. 341). As will be shown later, passive immunity can also be conferred on monkeys, and there is no doubt but that the same result can be obtained in man.

The quantity of blood which is to be used to induce passive immunity obviously depends upon the strength of such blood, that is, the number of units it contains, and upon the duration expected of such immunity. Thus, in mice, monkeys, and man, owing to the peculiar

conditions which bring on relapses, it is necessary to inject a much larger dose of immune blood than what would seemingly be necessary to protect against a simultaneous injection of the spirillar blood. While the calculated dose will prevent the first paroxysm it will not be sufficient to hold in check the first relapse, which appears on the fifth to seventh day after the inoculation. Hence, the need of injecting much larger doses of immune blood than such as are calculated from the experiments on rats in which no relapse occurs.

The duration of passive immunity depends, therefore, primarily upon the dose of the immune blood which has been administered. As seen from the following experiment, it may persist in rats for more than 68 days, and in mice for about 65 days.

Rat 5/1 received on February 16 a simultaneous injection of 0.1 c.c. of spirillar blood plus 0.010 c.c. of immune blood (result of 13 injections). No infection. Tested on March 31 with another injection of 0.1 c.c. of spirillar blood it was found to resist. Duration, 43 days.

Rat 4/245 received on February 8 a simultaneous injection of 0.1 c.c. of spirillar blood plus 0.050 c.c. of immune blood (13 injections). No infection resulted. Tested on March 31 with same dose as preceding; result negative. Duration 51 days.

Rat 6/245 received on February 8 a simultaneous injection of 0.1 c.c. of spirillar blood plus 0.090 c.c. of same immune blood as above. No infection. Tested on March 31, the same as the preceding and with like result. Duration, 51 days.

Rat 4/214 received on January 22 a simultaneous injection of 0.1 c.c. of spirillar blood plus 0.2 c.c. of immune blood (result of six injections). No infection. Tested on March 31, the same as the preceding, it became infected. This shows that 0.2 c.c. of a weak solution protects for less than 68 days.

It should be noted that the above results pertain to the immunity which follows a simultaneous injection of spirillar and immune bloods. Such mixed immunity is probably more lasting than if only immune blood had been injected. No experiments with pure passive immunity have as yet been tried.

HEREDITARY IMMUNITY.

It is an interesting fact supported by a number of observations that the *Sp. Obermeieri* may pass from the mother to the fetus. Three such cases, reported by Albrecht in the early eighties, deserve special mention. In one, a seven-months-old fetus lived eight days after birth and on autopsy spirilla were found in the heart blood. In another case, the fetus, also seven months old, lived 76 hours

after birth and on autopsy likewise showed spirilla. In the third case, the fetus, seven and one-half months old, was born on the 17th day of the second pyrexia, but although it showed no spirilla it presented the characteristic enlarged spleen.

These facts are in striking accord with the numerous observations which have been made during the past year regarding the presence of *Sp. pallida* in congenital syphilis. The finding of *Sp. Duttoni* in the eggs of ticks is another illustration of a seemingly general fact that the actively motile spirilla are able to pass from parent to offspring. Not only is it possible to develop congenital or hereditary infection, but it becomes equally possible to acquire active, and probably even passive, immunity in this way.

The experimental evidence which we have to offer on this point pertains to the rat. It clearly establishes the existence of congenital or hereditary immunity and has, as will be readily seen, an important bearing upon the origin of the immunity with reference to tick fever which the native negro children apparently enjoy. The only explanation which Dr. Koch has been able to give for such cases is that they had been infected in early childhood. That a somewhat different explanation is possible will be seen from the following:

Experiment 1.—A female rat, 2/117, was inoculated with 0.25 c.c. of spirillar blood on November 29. On December 3 it received a second injection of 0.25 c.c. of spirillar blood. On December 7 it gave birth to several young. These were not examined for spirilla at the time but were allowed to grow up. On February 7, two months later, the three surviving young rats were inoculated, each receiving 0.1 c.c. of rich spirillar blood. They were then examined daily for five days in succession. Two of the young rats gave no evidence of infection. The third rat showed spirilla, about one per field, on February 9, but none before or after. Control rats developed the usual infection. Fully to appreciate this experiment it should be stated that young rats are extremely susceptible to infection. In these the spirilla appear early (within 15 hours) and in very large numbers (50 to 100 per field) and persist for four or five days. It is evident, therefore, that *two, out of the three, had complete immunity two months after birth*, and that a third developed but a very slight infection.

Experiment 2.—A rat, 5/145, was given an injection of 0.25 c.c. of spirillar blood on December 13 and on December 15 it was given a curative dose of immune blood (1 c.c.). As a result of the injection the spirilla disappeared from its blood within five hours. On February 16, 18, and 20 it was given 0.25, 0.5, and 0.5 c.c. respectively of spirillar blood. On February 22 it gave birth to young. Three of these were inoculated on March 22 (28 days later) with 0.1 c.c. of spirillar blood and did not develop an infection. *In this case, the immunity, active or passive, persisted for more than one month.*

Experiment 3.—Rat 3/157 received 5 c.c. of a Berkefeld filtrate on December 17, but no infection resulted. On February 10, it was given an injection of 0.33 c.c. of spirillar blood. This injection was repeated on February 13, 15, 18, and 20. On February 22 it gave birth to young. Three of these were tested on March 29, the same as the preceding. No infection followed. *The immunity, active or passive, lasted for more than 35 days.*

Experiment 4.—Rat 7/140 received on December 11 an injection of 5 c.c. of a Berkefeld filtrate and two days later it showed a very slight infection, only one spirillum being found. Unlike the preceding it received no other injection. On February 17, 68 days after inoculation, it had young. Of these four were tested on March 24 with 0.1 c.c. each of spirillar blood. In the blood of all four, the spirilla promptly appeared on the first day; on the second day they were 20 per field; on the third day, 50 per field; and in one of the four they were still present on the fourth day.

This experiment serves as a control on the previous ones in so far as it shows the severity of infection which the young rats undergo. It further shows that any passive immunity which they acquired from the mother, injected 68 days before their birth, had disappeared in the 35 days which followed.

The immunity of the mother, it may be added, was largely a passive one, as is seen from the very light infection which she exhibited.

The foregoing experiments furnish abundant proof that immunity may be acquired before birth and may persist for a month or more. The conditions of the tests, however, were such as to leave open two possibilities. First, the young may have had congenital infection *in utero* from which they recovered either before or after birth, in which case they possessed an active immunity. Or, second, they may have acquired passive immunity from the mother. In the first three experiments it may be assumed that the immunity was of the first type. A further study of this interesting problem is contemplated.

PREVENTION.

The prevention of spirillar infection is so easily accomplished in rats, mice, and monkeys as to leave no doubt about the equal efficacy of the method in man. An essential requirement is a very active immune serum. In all experiments made heretofore with the spirilla of man, geese, or chickens the process of immunization was not pushed very far. Usually either recovered blood was used or such as was obtained after several injections of spirillar blood. It will be seen from the following trial that in ordinary recovery the blood has a low immunity value, not over one or two units per cubic centimeter.

Experiment with decline blood.—Rat 4/243 was bled 60 hours after inoculation with spirillar blood, that is to say, at the close of the decline stage. In the blood, on standing, the spirilla died out in less than 30 hours. Six days later a young rat, 2/253, was given a simultaneous injection of 0.5 c.c. of this decline blood plus 0.1 c.c. of spirillar blood. The spirilla appeared on the following day; on the second day they were 20 per field; on the third day 100 per field; on the fourth day only dead spirilla, about 10 per field and in addition some small agglutination groups, were to be found. The decline blood used for this experiment clearly contained less than two units per cubic centimeter, and probably did not contain even one unit. While the blood of this rat was insufficient to protect against a simultaneous injection of spirilla it was effective in the same dose when given three days before the injection of spirillar blood.

Experiment with recovered blood.—The fact that this blood has a marked preventive action has been incidentally brought out heretofore on p. 341. In the latter case the recovered blood of Rat 3/77 was found to protect in a dose of 0.5 c.c. against a simultaneous injection of 0.1 c.c. of spirillar blood. A dose of 0.2 c.c. was almost sufficient to accomplish this result. In other words, this recovered blood contained more than two and less than five units per cubic centimeter. It therefore was considerably stronger than the decline blood mentioned above.

Experiment with hyperimmunized blood.—The facts shown above in connection with recovered blood are brought out with remarkable clearness when the blood of hyperimmunized animals is used. Experiments of this kind have been made with the blood of rats which received 6, 7, 8, 13, 20, and 26 injections of spirillar blood. It will be sufficiently illustrative to give a series of tests with the latter blood.

Rat 2/127, after receiving 26 such injections, was bled and its blood was used for a large number of trials of which the following will be of interest. Of this blood 0.05 c.c. was added to 4.95 c.c. of normal defibrinated rat blood, and the mixture was set aside for two hours at 36°. Of this mixture, portions of 0.1, 0.2, 0.3, 0.4, 0.5 c.c., corresponding to 0.001, 0.002, 0.003, 0.004, 0.005 c.c. of the original immune blood, were measured out into Hitchens' syringes and diluted with salt-citrate solution. To each syringe, 0.1 c.c. of spirillar blood was then added and the whole at once injected into a rat.

Rat 8/58 received the above mixture containing 0.001 c.c. of immune blood.

The following day it showed spirilla, about $\frac{1}{10}$ per field; the second day, five per field; the third day only one could be found, and none on the fourth day.

Rat 5/61 received the mixture containing 0.002 c.c. of immune blood. It showed no spirilla on the first or second day after inoculation; on the third day only one spirillum was found and none on the fourth day.

Rat 6/51 received the mixture containing 0.003 c.c. of the immune blood. At no time did it show spirilla in its blood.

Rats 7/61 and 9/58 received the mixtures containing 0.004 and 0.005 c.c. respectively of the immune blood. No infection occurred in either of these rats.

From the foregoing, it is evident that 0.002 c.c. of the hyper-immunized blood was practically sufficient to protect against a simultaneous injection of spirilla. This blood, therefore, contained 500 immunity units, which represent a considerable increase in the immunizing value as compared with the strength of the recovered blood (two to five units per cubic centimeter).

Similar preventive experiments were made with white mice and the results were equally definite though the possibility of relapse must be taken into consideration.

Mouse 5/214 received on January 22 a simultaneous injection of 0.1 c.c. of spirillar blood plus 0.25 c.c. of immune blood (result of six injections). No infection.

Mouse 6/214 received on the same date a similar injection, except that 0.5 c.c. of the immune blood was used. No infection. When tested on March 28 with 0.1 c.c. of spirillar blood it failed to become infected, showing that the duration of the passive, or rather, mixed immunity exceeded 65 days. In a similar experiment, under identical conditions, a rat, 4/214, which received only 0.2 c.c. of the immune blood, became infected at the end of 68 days. (See p. 350.)

In these two experiments no attempt was made to ascertain the occurrence of relapses. It was realized, somewhat later, that in an animal subject to relapses, a preventive inoculation will prevent the usual first infection, but, strange to say, it will not ward off the relapses which come on about the same as if the first attack had taken place. This is true when small doses are used, but with very large ones it may be possible to protect even against the relapses. The prevention of relapses will be discussed at the end of this chapter.

Monkeys.—Only one experiment has been made thus far with the monkey. The details of this test, together with the control, will be given under the next heading. It can be stated, however, that 0.35 c.c. of an immune blood protected against a separate and immediate injection of 0.25 c.c. of spirillar blood. As in the case of mice, the dose of blood, however, was not sufficient to ward off the relapse.

The most active blood which we have obtained protects a 100 g. rat in a dose of 0.002 c.c., or in other words, one unit protects 100 g. body-weight. The protective dose for a 5 kg. monkey would be 50 units or 0.1 c.c. of this immune blood. Similarly, the protective dose for a 75 kg. man would be 750 units or 1.5 c.c. of this immune blood. If serum had been used instead of blood it probably would have been twice as strong, and hence a correspondingly smaller dose would have been effective.

It may be added that a smaller dose than the above would have been sufficient to protect if given a day or two before the injection of spirillar blood. This is seen in the fact, mentioned above, that a recovered blood, which does not protect against 0.1 c.c. of spirillar blood in 0.5 c.c. dose when given simultaneously, readily does so when given two or three days before. On the other hand, the injection of 0.5 c.c. of a recovered blood four days previous to inoculation with spirilla failed to protect a rat, whereas when given simultaneously it protected perfectly. Furthermore, experiments have shown that immune blood will protect, if given at any time previous to the appearance of the spirilla in the blood. After that occurs curative doses of the immune blood must be used.

The prevention of relapses is obviously a matter of some importance. One method by which this can be accomplished is to inject large *cure*-*tive* doses of immune blood. From the work which we have done thus far this seems to be the only practicable procedure. Two other methods suggested themselves, namely, active and passive immunization with small doses of spirillar and immune blood. These methods have been tried on mice, but the result has not been as satisfactory as was anticipated. The details of this test are briefly as follows:

Six mice were injected on April 17 each with 0.1 c.c. of spirillar blood, 1 / 109. Two of these were reserved for controls. Two of the mice were given each two injections of spirillar blood after the disappearance of the spirilla from the first attack. These injections were made for the purpose of inducing, if possible, an active immunity during the apyretic stage. The other two mice were given each two injections of immune blood with the idea of conferring a passive immunity during the spirillum-free stage. Examinations were made daily until May 7.

Control Mouse 2 / 110 showed spirilla on April 18 and 19. On April 26, two spirilla were found in its blood indicating the first relapse.

Control Mouse 3 / 110 also showed spirilla on April 18 and 19. On April 26 it had the first relapse, only one spirillum, however, being found. Two spirilla were found on May 2.

Mouse 4/110 showed spirilla on April 18 and 19. On the 20th, and again on the 23d, it was given an injection of 0.25 c.c. of spirillar blood. It had a relapse on April 26, four spirilla being found, and again on April 28 when but one was detected.

Mouse 5/110 showed spirilla on the two days as before. It was then given injections of spirillar blood at the same time as the preceding. This mouse showed no relapse until May 3 when two spirilla were found.

Mouse 6/110 showed spirilla on the two days as before. On April 20 and again on April 23 it was given injections of 0.25 c.c. of immune blood. A relapse occurred on April 27 when 10 spirilla were found. Also on May 3 when two spirilla were found.

Mouse 7/110 showed spirilla on the two days as before. It was then given injections of immune blood, the same as the preceding. It showed no relapse.

From the above it will be seen that apparently one mouse was actively immunized and one passively immunized to the extent of preventing a relapse. The mildness of the first relapse in the other four mice, however, leaves open the possibility that a relapse occurred in the other two, but, owing to the scantiness of the spirilla, was overlooked. Further experiments are needed to determine the feasibility of preventing relapses by either of these two methods.

CURE.

In view of the rather weak strength of recovered blood, which, as shown under the preceding head, has a value of but two to five immunity units per cubic centimeter, it follows that such blood cannot be used to advantage as a means of prevention, much less as a cure, for relapsing fever. With hyperimmunized blood, however, things are entirely different since such blood, containing 500 units per cubic centimeter, can be readily obtained at present and, without doubt, under improved conditions this strength can be greatly surpassed. The corresponding serum would obviously have about twice the strength of the defibrinated blood.

The method of immunization heretofore employed has been to inject intraperitoneally, on alternate days, 0.25-1.0 c.c. of rich spirillar blood into a rat. Possibly more rapid immunization, together with a greater anti-bacterial strength of the blood, can be secured by giving larger injections (1-2 c.c.). Such experiments are now being conducted with the hope of increasing materially the immunizing value of the blood.

From results obtained it appears that when a large dose of blood is injected an appreciable interval must elapse before the next injection is made, in order to allow a full recovery of the phagocytes and a regeneration of the anti-bodies. When such injections are made in close sequence the strength of an immune blood may be actually decreased by more than one-half. Thus, in one test 20 rats were immunized, each receiving 26 injections of spirillar blood in doses of $\frac{1}{4}$ – $\frac{3}{4}$ c.c. Twelve of the rats were then bled and the blood was found to have a value of 500 immunity units per cubic centimeter. The remaining eight rats were continued under treatment, but instead of receiving small doses they were given on alternate days 1.0–1.5 c.c. When bled, after several such injections, the blood was found to have a strength of about 100 units.

As yet no attempt has been made to prepare a curative or preventive serum from any other animal than the rat. It is our intention to immunize a horse at an early date, the chief drawback to this procedure being the large number of rats which must be employed in order to secure an efficacious serum.

Obviously, as soon as it becomes possible to replace the use of spirillar blood by a pure culture of the organism the last difficulty in the practical production of a curative and preventive serum will have been overcome. The larger animals can then be immunized with as much, if not greater, ease than is now done in the case of diphtheria. We know to what extent it is possible to immunize a horse against the cholera vibrio, it being not at all difficult to obtain a serum which will protect a guinea-pig in a dose of 0.0001 c.c., and the experience with rats justifies the belief that this result can be equalled with *Sp. Obermeieri*, for at present our serum protects a rat in a dose of 0.001 c.c.

Cure of injected rats.—The following experiments in the treatment of infected rats are selected for the purpose of demonstrating the efficacy of the method.

Experiment 1.—Rats 5 and 6/145, weighing about 150 g. each, were injected with spirillar blood on December 13. Forty-three hours later an examination showed that each rat had spirilla in the blood, about one-third per field. This, it should be stated, represented the onset and not the decline stage. The first rat was then given 1.0 c.c. and the second 1.5 c.c. of an immune blood, 3/97, 2/98 (result of

eight injections). Re-examined *five hours later not a single spirillum could be detected* in the blood of either rat. This shows that a relatively weak blood, in a dose of 1.0 to 1.5 c.c., is able to cure the disease in the onset stage within a few hours.

Experiment 2.—Rats 4/173 and 1/174 were inoculated with spirillar blood on December 26. On December 28 each of the rats had about three spirilla per field. The first was given an injection of 2 c.c. of an immune blood, 4/154 (result of seven injections). An examination made *half an hour after the injection failed to reveal a single spirillum*. The second rat was given an injection of 3 c.c. of the same blood, and in this also not a single organism could be detected one hour after the injection. This experiment, it may be added, was performed before the Society of American Bacteriologists which held its meeting in Ann Arbor on that day.

Experiment 3.—The following trial shows the effect of the immune blood when employed in a dose insufficient to effect a full cure. Rats 2 and 3/154 were inoculated with spirillar blood on December 16. Two days later they each showed spirilla, about 10 per field. Accordingly each one received 1.0 c.c. of immune blood, 7/104 (result of eight injections); the effect was about the same in each rat. At the end of two hours the spirilla clearly showed the injurious action of the immune blood. Most of the organisms were agglutinated in groups of 5 to 50 cells. The motion was very sluggish and apparently they were dying off. The number had decreased to about 0.5 per field. This condition persisted for several hours after which recovery from the effects of injected blood began to manifest itself. This was noticeable in the increased motion of the spirilla and by the lessened number of agglutination groups. When examined about seven hours later, the spirilla had apparently increased in numbers to about one per field; they showed a normal activity and scarcely any indication of agglutination.

This experiment teaches that an injection of a small amount of immune blood, insufficient to cure at once, may cause a notable decrease in the number of spirilla and by doing so may materially modify the course of the disease. This fact will, without doubt, find an application in the treatment of the disease in man.

Experiment 4.—Rats 4 and 6/40 received on March 12 an injection

of spirillar blood. When examined 48 hours later the former had 40 and the latter had 20 spirilla per field. The former was then given 0.25 c.c. and the latter 0.5 c.c. of an immune blood, 2/127 (result of 26 injections). The details of this test are given on p. 334. It will be sufficient to state here that the dose was not sufficient to cure at once. The effect of the immune blood was marked and was indicated by the presence of dead spirilla, and especially by the agglutination phenomena. The spirilla recovered from the effect of the anti-bodies in about three hours.

The blood employed in this experiment had a strength of 500 units per cubic centimeter, that is to say, it protected in a dose of 0.002 c.c. It follows that 125 to 250 units are apparently insufficient to cure an infected rat weighing about 150 g. Both rats showed spirilla on the following day, though in very small numbers.

It is true that the injection of the immune blood in the two preceding experiments produced a marked effect upon the spirilla and even caused some decrease in their number, but this result was offset by the unusual persistence of the spirilla on the following or third day. Taken as a whole, the result of the last experiment was not as favorable as in the other trials where 1.0 c.c. of immune blood derived from rats which had received one-half or even a less number of injections was used.

The explanation of this apparent failure was soon found to be due to an "anti-complement" action. On neutralizing this action of the immune blood by means of normal rat blood, as described on p. 347, it was possible to effect the cure of two rats, 2 and 3/55, in one and two hours respectively by injections of 0.5 c.c. of this same immune blood.

Inasmuch as the rats which weighed from 100 to 150 g. were cured by 0.5 c.c. of immune blood (= 250 units) it will be seen that from 1.7 to 2.5 units per gram of body-weight represents the minimum amount needed to effect a cure in rats. It probably will hold true in general that about two units per gram of body-weight will effect an immediate cure. In a monkey, as will be seen, the disappearance of the spirilla was prompt after the injection of five units per gram of body-weight. On the two-unit basis a man weighing 75 kg. would require 150,000 units, which amount would be contained in 15 c.c. of a serum 10 times as strong as the one used in our experiments.

Cure of injected mice.—Unlike rats, as pointed out heretofore, the white mice are subject to a variable number of relapses, and in this respect they approach the monkey and man. It was therefore desirable to determine the effect of curative doses of immune blood on these animals. The number of such experiments has not been large, but it has been sufficient to show, first, that the spirillar infection can be cut short or “cured” within an hour; and, second, that the “cure” is not necessarily permanent, for relapses may eventually occur, the same as in untreated mice.

Experiment 1.—Mice 2 and 4/213 received an injection of spirillar blood on January 21. On the following day they showed spirilla; the former one, and the latter two, per field. They were then given an injection of 0.25 and 0.5 c.c. respectively of immune blood (result of six injections). In the former the spirilla decreased in an hour to about one-fourth per field; in two hours long spirilla or agglutinations were present and in 14 hours no organisms could be detected. On the other hand, the second mouse also developed agglutination groups and there was a temporary decrease during the first hour or two, but 14 hours later the organisms had actually increased to 10 per field and on the following day the mouse was dead.

At the time of this experiment the possibility of a relapse in a “cured” animal was not expected, and no examination of the first mouse was made until February 19, that is, 29 days after the original inoculation, when two spirilla were found in its blood. This, it will be seen, was the time for a fourth relapse. On the following day no organisms could be seen. The mouse was then examined daily, until March 7, but no other relapse was detected. On March 12 it received an injection of 0.1 c.c. of spirillar blood without becoming infected, thus showing that a condition of active immunity existed.

The following experiment shows conclusively the curative effect of an immune blood and the occurrence of relapse notwithstanding such treatment.

Experiment 2.—Mice 2, 3, and 5/244, received an injection of spirillar blood on February 7. When examined 28 hours later spirilla were present. The first and third had one per field; the second two per field; and the third one per field. They were then injected with 2, 1, 0.5 c.c. of an immune blood (result of 13 injections). The first

when examined one hour later showed no spirilla. It was not examined again until February 21, from which time until March 12 it was examined daily. On February 23 (time for second relapse) one spirillum was found. On February 27 again a single spirillum was met with.

In Mouse 3/244, examined one hour after the injection of immune blood, only two dead and two very sluggish ones could be detected. Re-examined two hours later and on the next day with negative result. The mouse died on February 10.

In Mouse 5/244, within one hour, no spirilla could be found and the examination on the next day was likewise negative. From February 21 to March 7, it was examined daily but no spirilla could be detected.

A control mouse, 4/244, was inoculated the same time as the above and was examined daily until March 7. It showed spirilla on February 8 (two per field); February 9 ($\frac{1}{10}$ per field); and again on February 16 ($\frac{1}{10}$ per field). It therefore presented but one relapse. This mouse as well as 2 and 5/244, used in the above experiment, were inoculated on March 12, each receiving 0.1 c.c. of spirillar blood. All three resisted infection, demonstrating that a condition of immunity had been established.

No experiment has been made to cure mice with stronger immune blood than that used above. It is possible that a more active blood would be effective in preventing relapses. Attempts at preventing relapses in mice, either by active or passive immunization, have been described.

Cure of infected monkeys.—The following experiments serve to demonstrate the preventive and curative action of immune blood in these animals. The immune blood used for Monkeys 1 and 2 was the combined yield of six rats which had received 26 injections of spirillar blood. This mixture in a dose of 0.002 c.c. was almost sufficient to protect against a simultaneous injection of 0.1 c.c. of spirillar blood. The rat, 7/90, which received this injection showed only two very sluggish spirilla 48 hours after inoculation, and only one per field on the following day, after which they disappeared. Inasmuch as 0.003 c.c. protected perfectly, the blood clearly contained from 330 to 500 units per cubic centimeter. The curative value of

this mixture was not determined in rats, but it probably was about the same as in other tests where immune blood resulting from 26 injections had been used. In such trials from 0.5 to 1.0 c.c. (250-500 units) was usually sufficient to cure, in one hour or less, a rat weighing 100 to 150 g.

Macacus rhesus, No. 1.—This was a small female and weighed but 1,670 g. On account of her small size she was selected for the first cure experiment. Since, as pointed out above, rats which weighed from 100 to 150 g. were cured by 0.5 c.c. of immune blood containing 250 units it follows that from 1.7 to 2.5 units per gram body-weight represent the minimum amount needed to effect a prompt cure. It was therefore decided to give the monkey five units per gram of body-weight or a total of 8,350 units, which amount was contained in 16.7 c.c. of the immune blood. With the object of increasing the severity of the test, and at the same time approximating the conditions in treatment of man, the inoculations of the monkeys were made subcutaneously and not intraperitoneally as had always been done with rats. The fresh spirillar blood, 2/90, used for the inoculation, contained about 40 per field and the dose was 0.25 c.c.

On the day following the injection no spirilla could be detected in the blood of the monkey. They were present on the second day, about one-half per field. On the third day the number had increased from one to five per field. The spirilla probably had not as yet reached their maximum number, since only 24 hours had elapsed from the beginning of the attack, but as the duration of an attack, according to Norris and his co-workers, is from two to four days it was decided to proceed with the treatment. The monkey was therefore given subcutaneously 16.7 c.c. of the immune blood which amount as shown above represents five units per gram body-weight. Owing to the quantity of the fluid, the injections were made in several places so as to favor rapid absorption.

The first examination was made exactly one hour after the injection of the immune blood. *It showed a total absence of spirilla.* Not a single spirillum alive or dead could be detected in the fresh blood and no evidence of the organism could be obtained from stained preparations. The monkey was then examined every hour, as shown in the table, and to insure accuracy, at each interval four fresh blood prepara-

tions were examined (two by each one of us). Stained preparations were also made at these hourly intervals, but neither the quadruplicate examinations of the fresh blood or of the stained specimens revealed a single spirillum. Daily examinations made in duplicate during the next three weeks failed to discover any evidence of spirilla. A relapse, however, occurred on May 6 when 10 spirilla were found. The temperature, it will be seen, remained about normal.

On the day following the injection the monkey showed a diarrhea which persisted for two days. At the same time an urticaria-like rash or blotches appeared on the eyelids and especially at the outer and inner canthi. Two day later the rash had perceptibly subsided; no other effect of the treatment was noted.

The temperature, it will be noted, dropped promptly after the administration of the immune blood. It did not reach the normal level for some days. This undoubtedly was due at first to the blood injected, for a slight febrile condition may be expected to follow injections of whole blood, especially when given in large doses, as in this case, where the fluid introduced corresponded to 1 per cent of the body-weight.

As seen from Table 1 a rise in temperature occurred on the fourth day of the cure. This at first was interpreted as indicative of a relapse and although no spirilla could be found it was decided to give the monkey a preventive inoculation. Accordingly 1 c.c. of the immune blood was given subcutaneously. The persistence of the fever and the absence of spirilla led to a suspicion of sepsis. Owing to the large quantity of corpuscles injected, large nodules had formed at the several places and one of these masses was found to be infected. The abscess was opened on the fifth and again on the sixth day. The temperature promptly dropped to normal and has remained so for the remainder of the period of observation.

The number of leucocytes before the infection of the curative dose was about one per field. During the next three hours following the injection it had increased to about two to five per field, and this condition persisted for three or four hours after which the number became normal.

This experiment, therefore, shows that an attack of 24 hours' duration in a monkey can be cured by a subcutaneous injection of immune

blood. The evidence of the curative result is seen in the disappearance of the spirilla from the blood in less than one hour; in the prompt fall of temperature, and in the mildness of a very late relapse.

Macacus rhesus No. 2.—This was a female which weighed 2,910 grams. It was used to demonstrate the preventive properties of the immune blood. For this purpose it was given a subcutaneous injection of 0.25 c.c. of the spirillar blood, 2/90 (40 per field). Following this it was given, likewise subcutaneously, but on the opposite side of the body, an injection of 0.35 c.c. of the same immune blood as used for the above experiment.

The calculated dose of this immune blood, on the basis of 0.003 c.c. per 100 g. of body-weight, was 0.087 c.c. That is to say, since one unit is necessary for every 100 g. of body-weight, the monkey would therefore require 29.1 units as the minimum to afford protection against a simultaneous injection. Four times the calculated minimum dose, that is 0.35 c.c., was given, in order to insure a definite and positive result. This amount, it will be seen, contains 116 units on the 0.003 c.c. basis, or 175 on the 0.002 c.c. basis. The former corresponds to four, and the latter to six, units per 100 grams body-weight.

The test, as carried out, was perhaps more severe than usual, owing to the separate and subcutaneous injection of spirillar and immune bloods. In the previous work on rats and mice the spirillar and immune bloods were mixed together, and hence were injected simultaneously. As pointed out heretofore, this mixing of the two fluids *in vitro* may weaken and kill many of the spirilla. In the experiment as carried out, this injurious action was avoided, and consequently it may be regarded as a severe test of the efficiency of the preventive treatment.

Macacus No. 1, served as a control for this experiment and, as shown above, it developed spirilla in its blood 48 hours after the injection, and these persisted until they were destroyed by the curative injection.

The blood of *Macacus* No. 2 was most carefully examined in duplicate (two observers) on each day after the injections were made. No spirilla were found on the first, second, third, and fourth days. On the fifth day only one spirillum was found after prolonged search, but none on the sixth day. On the seventh day the spirilla reappeared

and were fairly numerous, that is to say, they were about $\frac{1}{10}$ per field. The dose of immune blood administered clearly prevented the first attack, or at all events, delayed it for some days. It was insufficient, however, to ward off the retarded appearance or relapse of the disease.

It was decided to test the effect of a small curative dose of immune blood on this relapse. The monkey was accordingly given at 3 P. M. a subcutaneous injection of 7.3 c.c. of immune blood of two rats (result of 26 injections). This dose represents about 0.25 per cent of the body-weight, or more accurately, 1.25 immunity units per gram body-weight. In view of the fact, pointed out on p. 359, that 1.7 to 2.5 units per gram represent the minimum amount needed to effect a prompt cure, it is evident that the dose given in this case was too small to effect a prompt destruction of the spirilla. This actually proved to be the case. It will also be seen that this amount of immune blood was not sufficient to prevent the occurrence of a relapse.

As indicated in Table 2, at 9:30 A. M. the spirilla were about $\frac{1}{10}$ per field and the temperature was 40.6°. At 3 P. M., at the time the inoculation was made, the spirilla had all but disappeared, none being found in the fresh blood and but very few in the stained preparations, and the temperature had fallen to 40.2°. As this change really occurred before the injection was made it can in no wise be ascribed to the immune blood. When re-examined at 5:30 P. M., no spirilla could be found and the temperature had dropped to 39.6°. On the next morning, *although the temperature was normal*, 38.9°, five active spirilla were detected in the blood. The daily examinations, in duplicate, during the following 10 days proved negative. On the 14th day after the first appearance of the spirilla in the blood a severe relapse occurred. This is seen in the large number of spirilla (10 per field), although the temperature was not very unusual. From the table it will be seen that on the seventh and eighth days after the first appearance of the spirilla there was a marked rise in temperature, which might be regarded as indicative of an abortive relapse. At all events no spirilla could be detected in the fresh blood or in stained preparations. On these two days, it so happened that the temperature was taken in the afternoon and the elevation was regarded at the time as due to the tubercular condition.

A study of Table 2 will show that the immune blood has exerted

considerable preventive action. This is seen in the absence of a first attack, the mildness of the first relapse (April 11-14), and the absence of a second relapse on April 18-19. During the following week the anti-bodies apparently decreased considerably, for the relapse on the 25th was marked. In fact, the spirilla were more numerous than at any previous time.

The high daily temperature, especially that of the afternoon, together with a persistent diarrhea and some cough indicates a probable tuberculosis.

TABLE 1.
CURATIVE EXPERIMENT.
Macacus rhesus No. 1, Female, 1,670 Grams.

Date of Examination	Spirilla	Temperature	
April 6	Received 0.25 c.c. of spirillar blood subcutaneously
" 7	o	
" 8	$\frac{1}{2}$ p. f.	
" 9, 10:00	5 p. f.	41.0°	At 12:15 given s.c. injection of 16.7 c.c. of immune blood (5 units per gram body-weight)
" 11:15	o	40.2	Increased leucocytosis
" 12:15	o	40.2	" "
" 13:15	o	39.9	" "
" 14:15	o	39.7	Decreased "
" 15:15	o	39.5	Normal "
" 16	o	39.7	Diarrhea and rash
" 17	o	39.5	" "
" 18	o	39.6	" absent, rash less
" 19 9:30	o	40.2	" slight
" 3:00	o	40.6	Injected s.c. 1 c.c. of immune blood. Abortive relapse
" 5:30	o	40.5	
" 14	o	40.4	Abscess opened
" 15	o	40.05	" reopened. Slight diarrhea
" 16	o	39.3	No diarrhea
" 17	o	38.6	
" 18 P. M.	o	39.0	
" 19	o	39.0	
" 20	o	38.3	
" 21	o	38.3	
" 22	o	38.0	
" 23	o	38.95	Hemokonia present in variable amount from day to day
" 24	o	38.6	
" 25	o	39.1	Hemokonia common
" 26	o	38.6	
" 27	o	38.8	
" 28	o	38.3	
" 29	o	39.1	Hemokonia
" 30	o	38.4	
May 1	o	38.8	
" 2	o	39.0	
" 3	o	38.35	
" 4	o	38.1	
" 5	o	38.4	
" 6	Ten found	38.7	
" 7	o	39.1	

This experiment clearly shows that an immune serum in a dose of four to six units per 100 grams of body-weight was sufficient to prevent a first attack but not the relapse which came on in the usual time.

Furthermore, a dose of 1.25 immunity units per gram body-weight is not sufficient to insure a prompt destruction of the spirilla in a relapse. Neither is it enough to prevent the further recurrence of relapses. It is probable that the latter dose if given in the beginning would have been sufficient to prevent, not only the first attack, but also the relapse, and a dose approximately of this strength, will be desirable as a prophylactic in man.

TABLE 2.
PREVENTIVE AND CURATIVE EXPERIMENT.
Macacus rhesus No. 2, Female, 2,910 Grams.

Date of Examination	Spirilla	Temperature	
April 6, A.M.	Received separate s.c. injections of 0.25 c.c. of spirillar blood and 0.35 c.c. of immune blood (4-6 units per 100 grams body-weight)
" 7	o	
" 8	o	
" 9	o	
" 10	o	39.7°	
" 11	One found	39.6	Fifth day
" 12	o	39.5	
" 13 9.30.	10 p. f.	40.6	
" 3.00.	One found	40.2	Received 7.3 c.c. of immune blood (1.25 units per grams body-weight)
" 5.30.	o	39.6	
" 14	Five found	38.9	Diarrhea
" 15	o	40.25	No diarrhea
" 16	o	39.4	" "
" 17	9	39.1	" "
" 18 P.M.	o	41.2	Abortive relapse? or tuberculosis?
" 19. " "	o	40.6	" "
" 20	o	39.2	Chills and diarrhea
" 21	o	38.85	" "
" 22	o	38.0	Slight diarrhea
" 23	o	39.15	" "
" 24	o	38.9	" "
" 25	10 p. f.	40.0	Diarrhea. Relapse on 14th day after first recognition of spirilla (April 11)
" 26	2 p. f.	41.1	
" 27	o	38.9	
" 28	o	40.7	Leucocytosis
" 29	o	39.15	
" 30	o	38.85	
May 1	o	38.2	
" 2	o	38.7	
" 3	o	38.9	
" 4	o	39.0	
" 5	o	38.8	
" 6	o	39.2	
" 7	o	39.1	

General considerations.—The foregoing facts clearly show that an attack of relapsing fever can be aborted or cured in rats, mice, and monkeys within a very short time, in an hour or less, provided a sufficiently active immune blood or serum is employed. The experiments on monkeys indicate that a single curative injection may not only cause the prompt disappearance of the spirilla but may practically prevent the occurrence of a relapse. When a relapse does

occur after treatment, as in mice and in the monkeys it is seemingly milder than would otherwise be the case. Moreover, it is obvious that such relapses can be cured as easily as, and perhaps more so than, the original attack.

The prophylactic experiments on the animals mentioned, also show clearly that it is possible, with a relatively small dose, to prevent an attack of the disease. They also show that this preventive action is of short duration, for unless the quantity of serum used be quite large, relapses are likely to occur, even in the absence of the original or first attack, which may be looked upon as having been aborted by the prophylactic injection.

The prevention of relapses, it has been shown, can be accomplished by the injection of a single large *curative* dose which not only will destroy most of the spirilla at once, but also will remain long enough in the body to prevent the growth of any surviving organisms. As seen from the experiment with Monkey No. 1, a dose of five immunity units per gram of body-weight is sufficient to cure and prevent the subsequent relapse for a period of 27 days.

It may be possible to prevent a relapse by the injection of one or two small curative doses of immune serum (about one unit per gram of body-weight) two or three days before the relapse is due. In this way passive immunization may be brought about, and to such an extent, that the relapses can no longer occur.

Another possible procedure for the prevention of relapses consists in an active immunization of the animal during the apyretic stage. This can be done by injecting a large dose of spirillar blood immediately after recovery from an attack. The greater part, if not all, of the organisms thus introduced are at once destroyed, and the active immunity should be correspondingly increased. A day or two later a second injection of spirillar blood should be given still further to increase the immunity. This treatment may be repeated once or twice more before the close of the apyretic stage in order to obtain as great a resistance as possible. This procedure, it will be noticed, is essentially a vaccination with living virulent organisms and may be compared to the well-known anti-rabic treatment, in which progressive inoculations with a living virus are made previous to the appearance of the disease.

It will be seen that the methods of passive and active immunization can be combined into a prophylactic measure which will assure man a perfect and lasting protection against the disease. This vaccination will be as positive as that against small-pox. To secure this protection it will be necessary to receive, first, an injection of the immune serum (one to two units per gram of body-weight), and then, on the second and fourth days injections of spirillar blood. The active immunity which is thus induced in experimental animals is known to persist for several months, and without doubt it could be increased so as to last for a year or more.

A combined curative and preventive treatment will undoubtedly be found to be efficacious in the human disease. If only rat serum is employed, at most a very unsatisfactory procedure on account of the smallness of the animal, the quantity would hardly be sufficient to cure an attack. Thus, the dose for a 75 kg. man, corresponding to that used for Monkey No. 1, would be 750 c.c. or about 375 c.c. of serum. While the use of so large an amount of fluid is out of question it should be remembered that the strength of the serum used by no means represents the maximum obtainable. It is quite certain that one can be obtained which will be 10 times as strong, and with such the dose would be but 37.5 c.c., a quantity which is easily tolerated. On the two-unit basis the dose of such a serum would be but 15 c.c. Even stronger sera than this are possible, which, of course, would still further decrease the dose needed to cure an attack and at the same time protect against a relapse. For this purpose, as shown heretofore, at least five units per gram of body-weight are necessary.

The dose as mentioned is sufficient to cause the almost immediate disappearance of the organisms from the circulation. As rapid a result as this may not be desirable in the treatment of man, owing to the possible danger of thrombosis due to the agglutination of large numbers of spirilla. Smaller doses, repeated every hour or two, will probably be preferable to the use of a single large dose. The injection of about two units per gram of body-weight, repeated once or twice in the course of the day will probably offer the safest procedure.

The preventive dose for a 75 kg. man, corresponding to that

given Monkey No. 2, would be 9.0 c.c. of blood or about 4.5 c.c. of serum (six units per 100 grams of body-weight). Obviously, with a more active serum this dose can be materially decreased, but even as it stands it compares favorably with the quantity of diphtheria antitoxin administered for preventive purposes. The protection acquired by such a dose is of but short duration.

For the present it will be necessary to resort to the immunization of horses by injecting the spirillar blood of rats. When the artificial cultivation of *Sp. Obermeieri* is once accomplished it will be possible to immunize such animals with ease and to obtain large quantities of an extraordinarily active serum. The use of the serum or immunized rats is hardly practicable, though it may be found useful in localities where the disease is prevalent.

The experimental work here given will, without doubt, serve also as a basis for the serotherapy of tick fever which, notwithstanding its clinical resemblance to relapsing fever, must be considered as etiologically distinct from the latter.

AGGLUTINATION.

The presence of specific agglutinins can be readily demonstrated in decline as well as in recovered blood. When, for example, the decline blood which is still rich in spirilla is drawn, defibrinated, and placed in a test tube, the corpuscles soon settle, leaving a clear serum above. In the course of 12 to 24 hours a whitish film or patches appear above the layer of corpuscles. When one of these patches or islands is taken up with a finely drawn-out tube it will be found to consist of enormous masses or agglutinations which may fill several fields of the 2 mm. objective. The border of each mass is fringed with radially arranged spirilla. The smaller groups of 50 to 200 cells form perfect radiating rosettes.

At first the spirilla exhibit a very active motion, and while in this state the appearance of the rosette is extremely interesting. Gradually, however, the motion decreases, and eventually all the spirilla become immobilized, and not infrequently it will be found that the spirilla have completely died out in 24 hours. In such blood, it may be added, there is no evidence of granulation.

In onset blood, under similar conditions, the spirilla remain single or nearly so, and, being specifically lighter, they form a light-colored deposit above the surface of the corpuscles. No patches or islands form, thus indicating the absence of an agglutinating action.

The blood of a recovered rat may be expected to have a more intense agglutinating action than in the decline stage. That such is the case can be readily seen from the following experiment. The blood of Rat 3/77 was drawn 12 hours after the disappearance of the spirilla. Two drops of this blood were mixed with a like amount of fresh spirillar blood, the latter containing about 40 spirilla per field. The mixture, examined at once, showed the very long compound spirals of 50 μ or more in length, also agglutination groups of 3 to 20 clls. At the end of five minutes not a single free spirillum could be found. They had all gathered up into tangles of 10 to 50 very actively motile cells. At the close of 20 minutes, many of the spirilla were dead and granular débris was noted. No living organisms were present at the end of 50 minutes, at room temperature. In a similar mixture placed at 37°, the spirilla at the end of 30 minutes were all found to be dead and agglutinated into groups of 10 to 100 cells. In the control blood the spirilla remained single and active.

Hyperimmunized blood.—When highly immunized blood is added to spirillar blood in the proportion of 1:100 agglutination occurs almost instantly, as will be seen on reference to the data presented on p. 333. With such blood, however, agglutination can be brought about in much greater dilutions. Thus, in one experiment the following three mixtures were used:

	No. 1 1-1,000	No. 2 1-2,000	Control
Normal rat blood.....	0.8 c.c.	1.8 c.c.	0.45 c.c.
Dilute immune blood (0.1 cc.=0.001 c.c. original blood).....	0.1	0.1
Spirillar blood.....	0.1	0.1	0.05

Experiment 1.—With 1:1,000 mixture. This, when examined one minute later, showed hyperactive spirilla, also groups of three to four cells; five minutes later showed very long compound spirals, also loose tangles of 5 to 20 cells, likewise perfect rosettes; 20 minutes later showed in every field one to two small rosettes of five to 50 cells, all very active.

Experiment 2.—In this the 1:2,000 mixture was used. When examined one minute later showed hyperactive single and double spirals; five minutes later showed very long compound spirals, also loose tangles of 5 to 10 cells; 20 minutes later showed rosettes of 50 or more cells as in Experiment 1.

The control mixture even at the end of 30 minutes showed only single spirilla.

The foregoing tests merely demonstrate that the agglutinating action occurs *in vitro*. That a similar reaction may, and does occur *in vivo* can be easily shown. For this purpose all that is necessary is to inject a sufficient amount of an immune blood into a rat. Depending upon the amount injected, agglutination groups consisting of 10 to 100 or more cells promptly appear within a few minutes. If the amount of blood is insufficient to effect a cure these groups will persist for a greater or less length of time, two or three hours. The motion of the spirilla in such groups becomes sluggish and may cease altogether, so that it is not unusual to find groups of apparently dead spirilla.

An admirable demonstration of agglutination within the living body is seen when recovered, hyperimmunized, or passively immunized rats are given an intraperitoneal injection of rich spirillar blood. The results of such experiments are given under Pfeiffer's phenomenon.

It is worthy of note that irregular tangles of spirilla; living or dead, are also met with in the last stages of infection. These are not very common, and the perfect rosettes are only met with after the injection of immune blood or when decline blood is allowed to stand *in vitro* for some hours.

The agglutination phenomenon appears to begin with the union of two cells by means of their flagella. The cells may be brought close together forming a continuous long spiral or they may remain separated by the length of the whip (see Plate 9). Other cells may then attach themselves in like manner and thus give rise to a very long compound spiral. These may measure from 50 to 100 μ in length, and hence may consist of 10 or more cells. The tendency to form these long spirals is seen best when the amount of the agglutinins is small. When these are present in increased amount, either loose tangles composed of irregularly grouped cells or perfect, radiating rosettes result. It is probable that in the latter the flagella are directed centrally. Incidentally, it may be stated that rosette formation among bacteria has been described by Hefferan.

IDENTIFICATION OF SPIRILLA.

The question of the identity of the spirilla found in relapsing fever in different parts of the world is one that demands attention. In the absence of a cultivation method there remain but two other procedures, namely, animal inoculations and serum reactions. The animal reactions, particularly of rats and mice, can be made in almost any locality. Moreover, as shown heretofore, the viability of the spirilla in onset blood is such that the latter can be shipped to distant parts for further study and comparisons. Such onset blood, as has been shown in this instance, may contain infective spirilla for almost 40 days. It should be drawn under aseptic conditions and placed in a sterile test tube or in glass tubing which is then sealed in the flame. Presumably the blood or serum of hyperimmunized animals retains its specific properties for months and hence can be sent almost anywhere. Such a procedure is advisable when the living organism does not remain alive *in vitro* for any length of time.

The serum reactions which may be used for the purpose of identifying a spirillum will include the agglutinating, germicidal, and immunizing properties.

The agglutinating and the germicidal actions can be observed at the same time. The immune blood of known origin can be mixed in a watch-glass with a like amount of spirillar blood derived from man or an animal. Prompt agglutination and immobilization may be taken as proof of the identity of the organisms, especially when this occurs with dilute immune blood. This dilution can be made with normal blood or serum. Although we have made no experiments on this point, it is probable that killed spirilla may be employed for these tests in a manner analogous to the use of dead typhoid bacilli in the corresponding Widal test. Obviously onset blood must be used for such trials to avoid the auto-agglutination which otherwise is likely to occur.

The above tests which are made *in vitro* can be supplemented by a Pfeiffer's reaction. For this purpose the immune blood mixed with spirillar blood, is injected into the peritoneal cavity of a rat and from time to time some of the liquid can be removed by means of a capillary tube and examined for agglutinations and

dead spirilla. For final and complete identification it will be desirable to test the preventive and curative action of the known immune blood against the suspected spirillum. It is clear that the serum reactions are of the utmost importance in the identification of the pathogenic spirilla, and the suggestions here outlined are for the purpose of stimulating inquiry into the subject.

DIAGNOSIS OF RELAPSING FEVER.

The detection of spirilla in the blood is an easy matter when they are fairly numerous. At other times it requires a very careful search and a trained eye. This is particularly true when a living preparation is examined. The great motility of the organism and its extreme thinness renders it difficult of perception. Their presence is very often indicated by the sudden start of a corpuscle as a result of collision. The Welsbach light is much preferable to electric illumination or even to day-light, and in our work we have used it almost exclusively.

When very few spirilla are present it may be possible to detect them by a process of "enrichment." For this purpose the defibrinated blood is placed in a sterile tube and set aside for a day or two. The spirilla being lighter settle in a layer just above the corpuscles. With a capillary tube this layer can be withdrawn and examined. All the spirilla are thus concentrated in a small volume, and hence can be more readily detected. Centrifugation may be employed to accomplish this same result.

The staining of the spirilla is easily effected with any of the modifications of the Romanowsky method. The MacNeal method, in which pure Bernthsen insoluble methylene violet is used, gives an exceedingly heavy stain in a couple of minutes. The intensity is such that the organisms can be seen even with a No. 3 objective. Several of the photographs accompanying this paper are of preparations stained by this method.

The inoculation of rats, mice, and perhaps monkeys should be resorted to in order to secure material which will serve for a more complete identification of the organism. The blood used for this purpose should be drawn before crisis has taken place, owing to the

possibility of the immune bodies being present in the later stages in sufficient amount to prevent infection. The failure of some experiments in the past may possibly be due to the use of such blood.

The serum diagnosis may be resorted to, especially in cases which have recovered from the first attack or from a relapse. The blood should be drawn immediately, or shortly after an attack, for at that time it is richest in agglutinating, germicidal, and immune bodies. These may be notably decreased in amount just before the onset of a new attack and for that reason the blood should not be used at that stage. The test for agglutinating and germicidal agents can be carried out as given above. The immunizing action of the suspected blood can be tested in like manner, but for this purpose a relatively large dose of the blood must be used. At least 5 c.c. of such blood should be injected into a rat about 24 hours previous to the inoculation with the known spirillum. If the preventive action is to be tested on a monkey a much larger amount, about 50 c.c., should be used.

It will be seen that the serum diagnosis of the disease hinges upon tests with living spirilla which at present can only be maintained in a living animal. When artificial cultures are realized the diagnosis will be as easy as in the case of typhoid fever.

FILTRATION EXPERIMENTS.

The filtration of spirillar blood was suggested by previous experiments with *Tr. Lewisi* and *Tr. Brucei*. In the cultures of the former Novy and MacNeal noted very small forms, from 2 to 5 μ in length, and on filtering such material, previously diluted with salt solution, through a small Berkefeld filter under a pressure of five pounds, a perfectly clear liquid was obtained, which when injected into white rats caused a typical infection with *Tr. Lewisi*. Of nine experiments made with *Tr. Lewisi* three were positive, five were negative, and one was uncertain, since the control animal failed to develop an infection. Similar experiments with cultures of *Tr. Brucei* and with suspensions of the organs of animals infected with the latter gave negative results.

It will be shown in the following experiments that the spirilla pass quite readily through the small Berkefeld filter, especially under

the conditions which obtained. Owing to the importance of these experiments it is deemed best to give the details as fully as possible.

The spirillar blood was drawn from the rats two days after inoculation, at which time it was rich in very actively motile organisms. A new infected rat and a new filter were employed for each experiment. The defibrinated rat blood (2 c.c.) was diluted with 20 c.c. of a salt-citrate solution (0.5 per cent each), and this mixture was at once placed in the small brass cylinder which held the Berkefeld bougie. The cylinder was then closed with a screw cap which was connected with a tank containing air under a pressure of 50 pounds. The lower end of the bougie was connected by means of a short rubber tube, provided with a Hoffmann screw clamp, to a sterile receiver. This clamp was screwed tight before the liquid was poured into the cylinder and it was not opened until one or two minutes after the compressed air had been turned on. A clear reddish liquid slightly discolored by dissolved hemoglobin, instantly came through. The pressure was allowed to remain on for about three minutes, or until it had dropped to about 40 pounds.

The filtrate of each experiment was injected at once into three rats. At the same time it was examined in duplicate for corpuscles and for spirilla. In all cases these examinations were negative. The cylinder was then opened and a drop of the fluid on the filter was examined. This always contained an abundance of living, very actively motile spirilla.

The small Berkefeld bougie was used in this work. The filter is 35 mm. long and 15.5 mm. in diameter. As the central canal is 7 mm. in diameter this leaves about 4.2 mm. for the thickness of the wall. As stated above, a new bougie was used for each experiment. For the first five experiments the bougies were carefully shaved with sandpaper. That used for Experiment 1 was reduced to a diameter of 9.7 mm., which therefore left the wall with a thickness of only 1.4 mm. In the next four tests, the bougie was reduced to only 12 mm., leaving the wall about 2.5 mm. thick or a trifle over one-half the original thickness. In the last five experiments unshaved filters were used. All the filters before use were placed in distilled water and sterilized in an autoclav. The total duration of an experiment, from the time the mixture was made until the rats were injected, averaged about 10 minutes.

The results of these experiments are brought together in the accompanying table:

TABLE 3.
RESULTS OF FILTRATION.

	c.c. Filtrate Injected	No. of Spi- rilla Found and Day	Spirilla Absent on	
Exp. 1, Bougie 9.7 mm.—				
Rat 1.....	5	+1 3d.	4d.	Immunity lasts 66 days
Rat 2.....	5	+4 2d.	3d.	
Rat 3.....	4	+2 3d.	4d.	
Exp. 2, Bougie 12 mm.—				
Rat 1.....	5	+2 3d.	4d.	Immunity lasts 108 days
Rat 2.....	5	+2 3d.	4d.	
Rat 3.....	7	+1 3d.	4d.	
Exp. 3, Bougie 12 mm.—				
Rat 1.....	5	+1 2d.	3d.	
Rat 2.....	5	+1 2d.	3d.	
Rat 3.....	5	+1 2d.	3d.	
Exp. 4, Bougie 12 mm.—				
Rat 1.....	5	+2 2d.	3d.	
Rat 2.....	5	+2 2d.	3d.	
Rat 3.....	10	+2 2d.	3d.	
Exp. 5, Bougie 12 mm.—				
Rat 1.....	5	+2 2d.	3d.	
Rat 2.....	5	+2 2d.	3d.	
Rat 3.....	7	+2 2d.	3d.	
Exp. 6, Bougie 15 mm.—				
Rat 1.....	5	o		Reinoculated 5 days later with 0.25 c.c. = +3d. $\frac{1}{2}$ per field. Absent 4d. = +3d. $\frac{1}{2}$ " " " " = o
Rat 2.....	5	o		
Rat 3.....	7	o		
Exp. 7, Bougie 15 mm.—				
Rat 1.....	5	o		Reinoculated 4 days later with 0.25 c.c. = 3d. + $\frac{1}{2}$ per field Absent 4d. = +3d. 1 " " " " = 3d. $\frac{1}{16}$ " " " "
Rat 2.....	10	o		
Rat 3.....	5	o		
Exp. 8, Bougie 15 mm.—				
Rat 1.....	5	o		Reinoculated 4 days later with 0.25 c.c. = +4d. 2 spirilla = +4d. 2 " " = +4d. 3 " "
Rat 2.....	5	o		
Rat 3.....	10	o		
Exp. 9, Bougie 15 mm.—				
Rat 1.....	5	+1 3d.		Reinoculated 4 days later with 0.25 c.c. = o = o = +2d. 1 spirillum
Rat 2.....	5	o		
Rat 3.....	7	o		
Exp. 10, Bougie 15 mm.—				
Rat 1.....	5	+1 2d.	4d.	
Rat 2.....	5	+2 2d.	3d.	
Rat 3.....	8	+5 2d.	4d.	

A study of the foregoing table shows that, under the conditions of the experiment, spirilla passed through all of the shaved filters. It is noteworthy that the organisms did not appear in the rats until on the second and even third day after inoculation, and that none were present on the following day. The number of spirilla in the infected rats was very small, usually but one or two being found in the whole specimen. Fresh blood preparations were examined in this work. This slight infection is clearly due to the passage of immune bodies through the filter. The duration of the immunity, notwithstanding the slight infection, was quite considerable since, as shown, the rats resisted infection 66 and 108 days respectively.

The experiments with the unshaved filter are not as clean cut as the preceding, owing to the thicker walls. They show, however, an infection of four out of 15 rats employed in the set. Of the 11 rats which showed no spirilla in their blood, on subsequent inoculation with a large dose of spirillar blood two did not become infected, in other words they were immune, which indicates that these were probably infected by the filtrate, but that, owing to the small number of organisms, these were overlooked. This would indicate that six out of the 15 rats, if not more, had become infected by the filtrates.

Of the nine which became reinfected on subsequent reinoculation one showed the spirilla on the second day, five showed them on the third day, and three on the fourth day. The number of spirilla found in each specimen was small, usually about two or three, and none were found on the following day. In control rats inoculated with the same material the organisms invariably appeared on the second day and were numerous, 10 to 20 per field.

Here again, it is evident that an immune body passed through the filter, and with this fact in mind it will readily be seen that a small number of spirilla may have traversed at the same time, but owing to the presence of the immune body they were unable to multiply. That such inhibition exists is actually seen in Experiments 9 and 10, where in the three positive cases the spirilla were very scarce. Owing to the extreme difficulty in detecting the spirilla when present in such small numbers it is quite probable that they were present in some of the other rats.

The conclusion to be drawn from these observations is that, under the conditions of the experiment, spirilla can pass through the filter, but that, owing to the simultaneous passage of an immune body, they are unable to multiply or do so only to a very limited extent. Hence, the failure to infect a rat with the filtrate does not necessarily mean that the spirilla were held back by the filter.

Furthermore, in view of the fact that *Tr. Lewisi* and *Sp. Obermeieri* do pass through a bougie, it follows that the filtration test is not a proof, as it is usually regarded, of the ultramicroscopic size of the organisms which traverse such filters. Another example of an organism which can pass through a filter is the *Micromonas Mesnili* of Borrel, who also has noted the interesting fact that spiril-

lar forms, resembling but shorter than *Sp. Obermeieri*, are present in tap-water and also traverse the filter. These organisms multiply in the filtrate at 20° but not in broth at 37°.

TICK FEVER.

The demonstration by Ross and Milne, Dutton and Todd, Koch, and others that the tick fever of Africa is due to a spirillum raises the question of the identity of this organism with that of *Sp. Obermeieri*. In our preliminary note of January 13, we pointed out the probability that tick and relapsing fevers were two distinct diseases, due to different species of spirilla.

This view was based upon the behavior of the two spirilla in experimental animals. Thus in rats the organisms of the former were found by Dutton and Todd to persist in the blood for from three to nine days after inoculation, a condition quite different from that noted with our spirillum. Moreover, they showed that the spirillum of tick fever was usually fatal to monkeys. Thus in five out of seven monkeys death was clearly due to the infection produced by the ticks, while in the remaining two it may have been due to other causes. In the infected monkeys, the spirilla were constantly present or nearly so. In these respects the course of tick fever in monkeys is quite different from that of relapsing fever, where recovery is the rule, and, moreover, the spirilla persist for but a few days although relapses may occur. In a young rabbit the tick-fever spirilla were present for 10 days after inoculation, but in an adult none could be found. In a guinea-pig spirilla were found for two days after inoculation. In our own experiments with *Sp. Obermeieri* we were unable to infect two young rabbits by intraperitoneal inoculation. Norris, Pappenheimer, and Flournoy, however, on intravenous injection were able to find spirilla in the blood for one and two days respectively in two rabbits, while in two others none could be found. Similarly in guinea-pigs we have not been able to find spirilla in the blood for more than one day after inoculation, whereas the experiments of Norris and his co-workers with these animals were negative. The natural immunity of the guinea-pig to *Sp. Obermeieri* was well established by the work of Sawtschenko and Melkich.

It will be seen, therefore, that the tick-fever spirillum differs

markedly from that of relapsing fever in its effects on monkeys, rats, rabbits, and guinea-pigs.

Through the courtesy of Dr. J. L. Todd of the Liverpool School of Tropical Medicine we received some slides of the tick-fever spirillum, made from the blood of rats and monkeys. A comparison of these preparations with the corresponding ones of *Sp. Obermeieri* show conclusively that the two organisms are entirely distinct. In view of these facts we have reached the definite conclusion that, notwithstanding the clinical similarity, the two diseases are etiologically different. Accordingly, as mentioned in the beginning of this paper, we propose to designate the organism of tick fever as *Spirillum Duttoni*.

It will be seen that the view that these diseases are distinct entities is not in accord with that of Dr. Koch. Although he points out that tick fever is characterized by shorter attacks (less than three days) than the European relapsing fever; that the spirilla in the blood of the former are exceedingly scanty as compared with the number found in the latter, he concludes that the diseases are not different, and that at most one can speak of an African variety of relapsing fever.

The morphological characteristics and the effects on animals offer at present the only evidence of the distinctness of these organisms. They are, however, sufficient to justify the conclusion reached and a belief that a full confirmation will be afforded when cross-experiments are made with the sera of animals immunized against these two spirilla.

In their preliminary note published March 10, Breinl and Kinghorn present important evidence bearing upon the individuality of tick fever. They show that the organism multiplies enormously in rats, and may produce death, the duration of the disease varying between one and 45 days; three or four relapses were noted before the rats died. Of six mice inoculated four died on the following day and the remaining two after 48 hours. *Sp. Obermeieri* is certainly not fatal to mice and rats, and in the latter the infection is short and without relapses.

Guinea-pigs showed only a temporary presence of spirilla, possibly due to a survival of the injected organisms. The injection of large

quantities of spirillar blood into rabbits caused death in from 3 to 10 days. In a dog a large dose caused a temporary infection, the spirilla being present for three days. A similar result was obtained with a pony. In one monkey inoculated with 1 c.c. of spirillar blood the organisms appeared within 24 hours, but disappeared after three days; subsequent relapses were noted.

As a result of these experiments on animals Breinl and Kinghorn reach the conclusion, the same as the one which we published two months before, that the two spirilla are different.

Morphologically, as stated, a very great difference exists between our *Sp. Obermeieri* and the spirillum of tick fever. The single individual or cell of *Sp. Duttoni* measures $16\ \mu$, and is hence about twice as long as that of *Sp. Obermeieri*. Similarly the pair in process of division or agglutination measures $32\ \mu$ in the case of *Sp. Duttoni*, while it is $16\ \mu$ long in the case of *Sp. Obermeieri*. Obviously in taking measurements of spirilla, care must be taken to discriminate between a single cell and an agglutination or compound spiral consisting of 2 to 10 cells. The ordinary statement that *Sp. Obermeieri* varies from 10 to $40\ \mu$ and more in length gives the erroneous impression that such measurements refer to a single cell, which is far from being the case. The illustrations accompanying this paper will show spirals, single and in pairs, also a composite consisting of many cells. Forms such as the latter may be found to measure anywhere from 50 to $100\ \mu$ and more.

The number of turns is likewise given in an indefinite way, as ranging from 6 to 20, and for that reason such figures can hardly serve as a basis of comparison. It is evident that the number of turns stand in relation to the length of the cell, that is, whether it is single or elongated prior to or in the act of division. The short cell resulting from division is about $8\ \mu$ long and has but two or three turns, whereas the dividing form averages $16\ \mu$ in length and has four to six turns. Our photographs will permit verification of these figures.

The single cell of *Sp. Duttoni*, on the other hand, measures $16\ \mu$ in length, and, notwithstanding that it is twice as long, it has but two or three turns, the same number as the shorter *Sp. Obermeieri*. It follows that the distance between the turns in the former is from 4 to $5\ \mu$, while in the latter it is but $1.5\ \mu$.

A further important characteristic is afforded by the width of the turn or of the whole spirillum. In the case of *Sp. Obermeieri* this width measures quite constantly $1.0\ \mu$, whereas in *Sp. Duttoni* it amounts to 2.0 to $2.7\ \mu$. This fact is referred to by Wellman when he says that the spirillum studied by him in West Africa "dies in rounder and more flowing curves than are generally described and pictured." His photograph, it may be added, is perfectly typical for *Sp. Duttoni* except in one regard, and that is the large number of spirilla which are shown in one field. Dr. Koch also speaks of the tick spirillum as appearing to be a trifle longer than the European spirillum. He regards the whiplike forms with long turns as the result of stretching incidental to the rapid drying of the very thin smears.

According to Dr. Koch, the spirillum of tick fever shows very little progressive motion, and hence remains at the same place for a long time. This certainly is not true for our organism, which moves about at great speed, especially in onset blood.

The number of spirilla which are present in the blood of man affords another distinction of importance. In the European and Asiatic relapsing fevers the spirilla are usually present in very large numbers, several being found in each field of the 2 mm. objective. In tick fever the spirilla are usually rare, and particularly is this true after the first attack. This scarcity of the spirilla was noted by the discoverers, Ross and Milne. In some films they found but two or three spirilla, while in one case, in which they mention the organisms as being "fairly numerous" the preparation showed but one spirillum in 30 fields.

Another feature to which attention should be called is the marked tendency of *Sp. Duttoni* to curl up thus giving rise to figure-8 forms or even perfect circles (Plate 12, Fig. 5). This tendency does not seem to exist in our spirillum.

A further distinction is afforded by the presence of several divisional zones in *Sp. Duttoni*, as a result of which this organism apparently yields a number of comma or S-shaped segments. This feature is shown in Plate 12, Fig. 4. Nothing of this kind can be observed in the *Sp. Obermeieri* which we have studied, but it should be noted that similar divisions are present in the spirillum of relapsing fever from Bembay.

The facts deduced above show conclusively that *Sp. Obermeieri* and *Sp. Duttoni* are two distinct organisms, and hence that the tick fever of Africa is distinct from the ordinary relapsing fever.*

BOMBAY RELAPSING FEVER.

The important question arises as to the identity of the spirillum studied by us with that described by Obermeier. The description given by him is necessarily meager and in only one respect is it possible to make a comparison. This is in the matter of length, which he gives as varying from the diameter of a corpuscle to 10 to 30 μ . As mentioned above, the length of our single cell, is 8 μ (about the diameter of a corpuscle, as can be seen in some of the photographs), while the pair is 16 to 18 μ long. The compound threads may, of course, be much longer. Obermeier gives no details as to the number of turns, and in the absence of illustrations it becomes difficult, if not impossible, to identify the species from his description.

We have been unable to make any direct comparison with European material, but through the kindness of Capt. W. S. Patton, I. M. S., we have received several blood smears from cases of relapsing fever occurring in Bombay.

A study of these slides revealed the interesting fact that the Bombay spirillum was quite different from our organism and that in some respects it resembled that of tick fever. It differs, however, from each of these to such an extent as to give rise to the belief that we are dealing with three distinct organisms. In other words, the evidence on hand points to the existence of three relapsing fevers in man. As to the existence of two of these there can be no doubt, but with regard to the third, that of Bombay, there may still be some question. In the absence of fresh material we are not in a position to settle this point, and such observations as we have made are here given with the object of directing attention to a possible plurality of the pathogenic spirilla. It becomes, therefore, very desirable to examine closely into the nature of the relapsing fever as met with in Russia, Asia, and Africa. It is not unlikely that a fair-sized group

*Since the writing of this paper one additional and clinching proof of the distinctness of these organisms has been supplied by Zettnow. In a short note of March 8 he shows that *Sp. Duttoni* has diffuse flagella. This observation effectually differentiates it from *Sp. Obermeieri*, which, as seen from our photographs has but one single terminal flagellum, and places it in the same group with *Sp. gallinarum*.

of spirillar infections will eventually be recognized. The necessity of resorting to the serum reactions, previously discussed, for the identification of spirilla will be readily seen.

The measurements of the Bombay spirillum, as seen from the subjoined table, agree fairly well with those of *Sp. Obermeieri*. There are, however, several notable differences as will be observed on comparing Plates 8 and 13. It will be seen that the Bombay spirillum is apparently thinner, that there is greater flexibility, a marked absence in the regularity of the turns, and considerable variation in their width. The agglutination into a long continuous spiral is not met with; the long forms, such as they are (Plate 13, Fig. 5), consist of several cells which are, rather loosely apposed. This fact may be taken to indicate the presence of diffuse flagella which presumably would not permit the perfect union seen in *Sp. Obermeieri* (Plate 9, Fig. 3) which is due, as has been pointed out, to the presence of a single end flagellum.

Another striking feature is the tendency of the spirillum to form loops, either circular or figure-8 forms, similar to those of *Sp. Duttoni*. This is brought out clearly in the agglutination (Plate 13, Fig. 6) which, it will be seen, consists largely of looped spirals. This tendency is also seen in the curled ends which Fraenkel and Pfeiffer aptly compare to a "Schweineschwänzchen." We have never noticed this peculiarity in the organism studied by us.

A further and very important characteristic is that of multiple transverse division, much as in the case of *Sp. Duttoni*. The long spirals not only show a median clear space, but each half in turn may show a similar clear zone. The resulting segments or units, as shown in Plate 13, Fig. 3, have an elongated S-shape. In some instances (Plate 13, Fig. 4) evidence of further division is presented resulting in comma or vibrio forms. The presumption is that these transverse bands represent division but it should be stated that no free short forms of the vibrio or S-type have been found in the blood. It is of interest to note that Carter as early as 1877 described the Bombay spirilla as dividing in two or three parts.

SPIRILLUM GLOSSINAE, N. SP.

This organism was met with in smears of the stomach contents of two out of six tsetse flies (Nos. 6 and 8, *Glossina palpalis*) which were received from Lieutenant A. C. H. Gray of the Sleeping Sickness Commission. The spirilla were present in small numbers. They were found as single short forms, $8\ \mu$ in length, or as double cells which measure about $15\ \mu$. It will be seen from the measurements given in the table, as well as from the photographs shown in Plate VII, that this organism is shorter and narrower and has more turns than *Sp. Obermeieri*. It will be noticed that the ends become indistinct and tapering. In some instances the spirals are drawn out into rather straight forms. In this species, as in the other spirilla studied, evidence of transverse division may be obtained (Plate 14, Fig. 4).

This occurrence of true spirilla in an insect is a matter of considerable interest. Up to the present time but three or four other instances are known. These are (1) *Sp. gallinarum* in the tick *Argas miniatu*s; (2) *Sp. Duttoni* in the tick *Ornithodoros moubata*; and (3) *Sp. Obermeieri* in the common bed-bug *Acanthia lectularia*. Recently (Februäry, 1906) the Sergeants reported finding spirilla in a preparation, made in 1901, from the gut of a larva of *Anopheles maculipennis*. They were found in considerable number, at times were agglutinated, showed 1.5 to 4 turns and measured 8.5 to $17\ \mu$. It is barely possible that this organism is the same as our *Sp. glossinae*.

TABLE 4.
MEASUREMENTS OF SPIRILLA.

	Length in μ	Width of Filament	Number of Turns	Distance of Turns	Width of Turn or of Spiral
<i>Sp. Obermeieri</i> —					
Single cell	8.0	0.25	2-3	1.5	1.0
Double cell	16-20	4-6	2.5
<i>Sp. Bombay</i> —					
Single cell	8.0	0.2	2-3	2-3	1.0-1.3
Double cell	16-20	4-5
<i>Sp. Duttoni</i> —					
Single cell	16	0.2	2.5	4-5	2.0-2.7
Double cell	30	5-6
<i>Sp. Glossinae</i> —					
Single cell	8.0	0.2	4.0	1.3	0.6
Double cell	15.0
<i>Sp. pallida</i>	13-15	0.2	11	1.3	0.6
<i>Sp. gallinarum</i>	10-12	0.25	4.0	2.0	1.0

The measurements given in this table are made from photographs magnified 1,500 or 3,000 times.

The *Spirochaete Ziemanni*, which Schaudinn reported as a stage in the life-history of the intracellular parasite *H. Ziemanni*, as has been shown, is not a spirillum but a trypanosome. In our paper on bird trypanosomes we questioned the relation of this trypanosome to the cytozoon of the owl. Since then we have shown that flagellate infection of mosquitoes is not an uncommon occurrence, and, in addition, we have obtained evidence which conclusively demonstrates that the organisms in question are in no wise related to *H. Ziemanni*.

SUMMARY.

The main results of this extended study can be condensed into the following statements.

1. *Spirillum Obermeieri* belongs to the bacteria and not to the protozoa.

2. In onset blood, kept *in vitro*, it may be maintained alive for 40 days, whereas in decline blood, owing to the presence of a germicidal agent, it rapidly dies out.

3. Man, monkeys, white mice, and rats, tame and wild, are subject to infection. The first three are subject to relapses, the latter are not.

4. All attempts at cultivation have proved unsuccessful.

5. A powerful specific germicidal body is present in decline and in recovered blood, notably in blood of hyperimmunized rats. This body does not originate after the blood is drawn, but exists within the living animal.

6. An immunizing body is also present and is probably distinct from the germicidal agent.

7. Pfeiffer's phenomenon can be demonstrated *in vitro* and *in vivo*. In the peritoneal cavity of hyperimmunized rats, the spirilla are killed almost instantly, after which they are taken up by the macrophages or large mononuclear cells.

8. Active immunity follows recovery from the infection. By successive injections of spirillar blood this immunity can be increased to a remarkable degree.

9. Passive immunity can be imparted by injections of recovered or hyperimmunized blood.

10. Both active and passive immunity may last for months.

11. Hereditary immunity can be obtained and is probably the result of infection *in utero*.

12. Preventive inoculations can be successfully made in rats, mice, and monkeys.

13. Infected rats, mice, and monkeys can be promptly cured by injection of hyperimmunized blood. Subsequent relapses, if any, can be prevented by curative doses of blood.

14. The preventive dose should be about 10 immunity units per 100 grams body-weight.

15. The curative dose is about five immunity units per gram body-weight.

16. A solid basis is thus established for the prevention and cure of relapsing fever in man.

17. Agglutination of spirilla occurs *in vitro* and *in vivo* under the influence of recovered or hyperimmunized blood. To a slight extent it occurs during crisis.

18. The agglutination, germicidal, and immunizing properties of recovered blood can be used in the sero-diagnosis of relapsing fever. Also for the identification of spirilla.

19. *Sp. Obermeieri* can be made to pass through a Berkefeld filter.

20. The tick fever of Africa is distinct from the relapsing fever of Europe. Its cause is *Sp. Duttoni*.

21. The spirillum of the relapsing fever of Bombay is apparently different from *Sp. Obermeieri* and *Sp. Duttoni*.

22. The evidence points to the existence of a group of relapsing fevers.

23. True spirilla occur in the stomach and intestines of insects.

24. The demonstration that *Sp. Obermeieri*, *Sp. Duttoni*, and *Sp. gallinarum* are bacteria and not protozoa means that many, if not all, of the other spirochetes belong to this same group.

25. The difference in number and arrangement of flagella may lead to a division into sub-genera.

26. The transmission of spirillar diseases by insects, and the congenital infection of mammals and eggs of insects are properties which up to the present have been regarded as characteristic of protozoa. These properties are now known for the first time to be shared by this group of bacteria. Yellow fever presents a marked analogy to the

spirillar infections and it is not improbable that the cause of this disease will be found to belong to this group of organisms.

Lastly, we desire to express our obligations to Dr. Charles Norris for the very great courtesy shown in placing the *Spirillum Obermeieri* at our disposal. Also we desire to acknowledge the kind assistance rendered by Dr. J. L. Todd, of Liverpool, Captain W. S. Patton, I. M. S., of Madras, Lieutenant A. C. H. Gray, of the Sleeping Sickness Commission, and by Dr. W. J. MacNeal. Our thanks are also due to the Board of Directors of the Rockefeller Institute for Medical Research for financial assistance rendered.

LITERATURE.

- ABEL, R. "Über das Vorkommen feiner Spirillen in Dejektionen Cholerakranker," *Centralbl. f. Bakt.* (I), Feb., 1894, 15, pp. 213-16.
- ALBRECHT, R. "Beitrag zur Kenntniss u. Entwicklung d. *Spirochaete Obermeieri*," *Deutsch. Archiv f. klin. Med.*, 1881, 29, pp. 77-112; also, in *St. Petersburg. med. Wchnschr.*, 1878 et seq.
- AUFRECHT. "Über den Befund feiner Spirillen in den Dejektionen einer unter Cholerasymptomen gestorbenen Frau," *Centralbl. f. Bakt.* (I), March, 1894, 15, pp. 405-7.
- BARDACH, J. "Recherches sur la fièvre récurrente," *Ann. de l'Inst. Pasteur*, 1899, 13, pp. 365-84.
- BALFOUR, A. "A Haemogregarine of Mammals and Some Notes on Trypanosomiasis in Anglo-Egyptian Sudan," *Jour. Trop. Med.*, March 15, 1906, p. 90.
- BIZZOZERO, G. *Centralbl. f. Bakt.* (I), 1893, 13, p. 623.
- BONHOFF, H. "Die *Spirochaeta vaccinae*," *Berl. klin. Wchnschr.*, Sept. 1905, pp. 1142-44, 9 figs.
- BORREL, A. "Microbes des eaux et culture d'un protozoaire minimal," *Compt. rend. de la Soc. de Biol.*, 1902, 54, pp. 61-62, 2 figs.
- . "Étude expérimentale de la clavelée," *Ann. de l'Inst. Pasteur*, 1903, 17, p. 128.
- . "Infections vermineuses et spirochetes chez les souris cancéreuses," *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 770.
- . "Cils et divisions transversales chez le spirille de la poule," *Compt. rend. de la Soc. de Biol.*, 20 janv. 1906, pp. 138-41, 2 figs.
- ET BURNET, ET. "Développement initial *in vitro* du spirille de la poule," *Compt. rend. de la Soc. de Biol.*, 17 mars, 1906, 60, pp. 540-42.
- ET MARCBOUX. "Argas et spirilles," *Compt. rend. de la Soc. de Biol.*, Feb. 1905, 58, pp. 362-64.
- BREINL, A., AND KINGHORN, A. "Observations on the Animal Reactions of the *Spirochaeta* of the African Tick Fever," *Lancet*, March 10, 1906, p. 668.
- CANTACUZÈNE, J. "Recherches sur la spirillose des oies," *Ann. de l'Inst. Past.*, July, 1899, 13, pp. 529-57, 2 plates.
- CARLISLE, R. D. *Vide* this number.

- CARTER, H. V. *Deutsche med. Wchnschr.*, 1879, pp. 189, 351, 386; also *Medico Chirurg. Trans.*, II Ser., 45, 1880.
- CASTELLANI, A. *Brit. Med. Jour.*, 1905 (2), pp. 1280, 1330, 1430; also *Deutsche med. Wchnschr.*, 1906, pp. 132-34.
- COHN, F. "Zur weiteren Kenntniss des Febris recurrens u. d. Spirochaeten," *Deutsche med. Wchnschr.*, 1879, pp. 189, 351, 386.
- . "Untersuchungen über Bacterien," *Beiträge zur Biologie der Pflanzen*, 1875, Bd. I, Heft III, p. 196, figure by Weigert.
- DUTTON, J. E., AND TODD, J. L. "The Nature of Tick Fever in the Eastern Part of the Congo Free State, with Notes on the Distribution and Bionomics of the Tick," *Memoir XVII, Liverpool School of Tropical Medicine*, Liverpool 1905; also in *Brit. Med. Jour.*, Nov. 11, 1905, p. 1259.
- EHRENBERG, G. *Die Infusionsthierchen*, Leipzig, 1838, p. 83; also, *Abhandlungen d. Berl. Akad.*, 1833, p. 313.
- ESCHERICH. "Notiz zu dem Vorkommen feiner Spirillen in diarrhöischen Dejectionen," *Centralbl. f. Bakt.* (I), 1894, 15, p. 408.
- FRAENKEL, C., AND PFEIFFER, R. *Mikrophotographischer Atlas der Bakterienkunde*, Hirschwald, 1892. Plate LXVI.
- GABRITSCHESKY, G. "Les bases de la sérothérapie de la fièvre récurrente," *Ann. de l'Inst. Past.*, 1896, 10, pp. 629-59.
- . "Réponse à M. Metchnikoff," *Ann. de l'Inst. Past.*, 1897, 11, pp. 238-44.
- . "Beiträge zur Pathologie u. Serothérapie d. Spirochäten-Injektionen," *Centralbl. f. Bakt.* (I), 1898, 23, pp. 365-71; 439-45; 635-41; 721-30; 778-82.
- GERHARDT. *Verhandl. d. phys.-med. Gesellsch. in Würzburg*, 1881, 15.
- GORSLINE, C. S. "On the Preparation and Use of Collodium Sacs," *Contributions to Medical Research*, dedicated to V. C. Vaughan, George Wahr, Ann Arbor, 1903, p. 390.
- GRASSBERGER, R. "Befund massenhafter feiner Spirillen in den Darmentleerungen einer an Cholera nostras erkrankten Person," *Wien. klin. Wchnschr.*, 1894, 7, p. 943.
- HEYDENREICH, L. *Klinische u. microscop. Untersuchungen üb. d. Parasiten des Rückfalltyphus u. s. w.*, Berlin, Hirschwald, 1877.
- HÖDLMOSE. "Die Serodiagnose des Rückfalltyphus," *Zeitschr. F. Heilkunde*, 1905, 26.
- IWANOFF, N. A. *Zur Frage v. d. künstl. Immunität bei Rückfalltyphus*, Dissertation (Russ.), 1897.
- . "Ein neuer Beitrag zur Phagocytenlehre: Die Phagocytose beim Rückfalltyphus," *Centralbl. f. Bakt.* (I), 1897, 22, pp. 117-19. 1 plate.
- . *L'Indépendance médicale*, Juin 17, 1896.
- JAMES, S. P. "Oriental or Delhi Sore," *Scientific Memoirs by the Officers of the Med. a. Sanit. Departm. of the Gov. of India*, New Series, 1905, No. 13, 16 pp. 1 plate.
- KARLINSKI, J. "Zur Kenntniss des fieberhaften Icterus." *Fortschr. d. Med.*, 1890, pp. 160-75.
- . "Zur Aetiologie des Recurrenstyphus. *Centralbl. f. Bakt.* (I) 1, 1902, 31.
- KOCH, R. *Deutsche med. Wchnschr.*, 1879, p. 327; also quoted by Cohn, *ibid.*, p. 189.
- . "Untersuchungen über Bakterien VI," Cohn's *Beiträge z. Biol. d. Pflanzen*, 1877, 2, p. 431, 2 photographs.

- KOCH, R. *Mittheilungen a. d. kais. Ges. d. Gesundh. a. d. Kaiserl. Gesundheitsamte*, 1881, 1, Photographs, Plate IV.
- . "Vorläufige Mittheilungen über die Ergebnisse einer Forschungsreise nach Ost-Africa," *Deutsche med. Wchnschr.*, Nov. 23, 1905, pp. 1865-69, 24 figs.
- . "Über afrikanischen Recurrens," *Berl. klin. Wchnschr.*, Feb. 12, 1906, pp. 185-94, 10 figs.
- KOWALSKI, H. *Wien. klin. Wchnschr.*, 1893, 6, p. 888; *Centralbl. j. Bakt.* (I), 1894, 16, p. 321.
- LACHMANN, B. "Klin. u. exper. Beobachtungen a. d. Recurrens-Epidemie in Gießen im Winter 1879-80," *Deutsch. Archiv f. klin. Med.*, 1880, 27, p. 50.
- LAVERAN, A. "Sur la Spirillose des Bovidés," *Compt. rend. de la Acad. d. Sc.*, 1903, 136, p. 939, 1 fig.
- ET VALLÉE. "Sur un cas de transmission par des ixodes de la spirillose et la piropalmoose bovines," *Compt. rend. de la Acad. d. Sc.*, 1905, 140, p. 1515.
- LEVADITI, C. "Contribution à l'étude de la spirillose des poules," *Ann. de l'Inst. Past.*, 1904, 18, p. 129, 1 plate.
- LÖVENTHAL, H. "Serodiagnose d. Febris Recurrens während d. Apyrexie," *Deutsche med. Wchnschr.*, 1897, Nos. 35 and 38; *ibid.*, 1898; also *Compt. rend. de XII Congrès internat. de méd. de Moscou en 1897*, Vol. 3 (1899).
- LUSTIG UND DE GIAXA, V. "Über das Vorkommen von feiner Spirillen i. d. Ausleerungen von Cholerakranken," *Centralbl. j. Bakt.* (I), 1894, 15, p. 721.
- MARCHOUX, E., ET SALIMBENI, A. "La spirillose des poules," *Ann. de l'Inst. Past.*, 1903, 17, p. 569.
- MARTIN, G. "Sur un cas de spirillose du cheval observé en Guinée française," *Compt. rend. de la Soc. de Biol.*, 1906, 60, pp. 124-26, 1 fig.
- MARTOGGIO, T., and CARPANO, M. "Spirillosi ovina (Nota preventiva)" *Ann. d'ig. sper.*, 1904, 14, pp. 577-82, 1 plate.
- MASSEY, A. Y. "Spirillosis in West Africa," *Jour. Trop. Med.*, Aug. 1, 1905, 8, p. 225.
- MELKICH, A. A. "Beiträge zur Pathogenese des Rückfalltyphus (Russ.)," *Arch. russ. de Pathol.*, 1900, 10; see also Sawtschenko.
- METCHNIKOFF, E. "Über den Phagocytenkampf beim Rückfalltyphus," *Virchow's Archiv*, 1887, 109, p. 176.
- . "Quelques remarques à propos de l'article de M. Gabritschewsky Sur la fièvre récurrente," *Ann. de l'Inst. Past.*, 1896, 10, p. 654; 1897, 11, p. 245.
- MILLER, W. D. "Über eine scheinbar pathogene Wirkung der Spirochaete dentium," *Deutsche med. Wchnschr.*, 1906, 32, p. 348.
- MOZUTKOWSKY, J. "Materialien z. Path. u. Therap. d. Rückfalltyphus," *Deutsch. Arch. j. klin. Med.*, 1879, 24, pp. 80-97; 192-221.
- . "Beobachtungen üb. Rückfalltyphus," *ibid.*, 1882, 30, pp. 165-81.
- NICOLLE, C., ET COMTE, C. "Sur une nouvelle spirillose (Note préliminaire)," *Compt. rend. de la Soc. de Biol.*, 22 Juillet, 1905, 59, p. 200.
- ET DUCLOUX, C. "De l'existence de la spirillose des oies en Tunisie," *Compt. rend. de la Soc. de Biol.*, 1903, 55, p. 1133.
- NORRIS, PAPPENHEIMER, AND FLOURNOY. *Vide this number.*
- NOVY, F. G., AND MACNEAL, W. J. "On the Filtration of Trypanosomes." *Sixth Report of the Michigan Academy of Science*, 1904, p. 180.
- . "On the Trypanosomes of Birds," *Jour. Infect. Dis.*, 1905, 2, pp. 256-308, 11 plates.

- OBERMEIER, O. "Vorkommen feinsten, eine Eigenbewegung zeigender Fäden im Blute von Recurrenskranken, Vorläufige Mittheilung," *Centralbl. f. d. med. Wissensch.*, 1873, pp. 145-47; also *Berl. klin. Wchnschr.*, 1873, pp. 152, 378, 391, 455.
- PALTAUF. *Wien. klin. Wchnschr.*, 1893, 6, p. 889.
- PATTON, W. S. "Notes on the Presence of Spirilla in a Tropical Ulcer," *Indian Med. Gaz.*, Feb. 1905.
- PETRIE, G. F. "Observations relating to the Structure and Geographical Distribution of Certain Trypanosomes," *Jour. of Hyg.*, 1905, 5, pp. 191-200, 1 plate.
- RECHTSAMER. "Über die feinen Spirillen in Dejektionen Cholerakranker," *Centralbl. f. Bakt.* (I), 1894, pp. 795-98.
- ROSS, P. H., AND MILNE., A. D. "Tick Fever," *Brit. Med. Jour.*, Nov. 26, 1904, p. 1453.
- SACHAROFF, N. "Spirochaeta anserina et la septicémie des oies," *Ann. de l'Inst. Past.*, 1891, p. 5, 564.
- SALOMON, H. "Über das Spirillum des Säugetiermagens u. sein Verhalten zu den Belegzellen," *Centralbl. f. Bakt.* (I), 1896, 19, pp. 433-42, 2 plates.
- SAWTSCHENKO ET MELKICH. "Étude sur l'immunité dans la fièvre récurrente," *Ann. de l'Inst. Past.*, 1901, 15, pp. 497-526.
- SCHAUDINN, F. "Generations- und Wirtswechsel bei Trypanosoma und Spirochaete. (Vorläufige Mitteilung)," *Arbeiten a. d. kaiserl. Gesundheitsamte*, 1904, 20, pp. 387-439, 20 figs.
- . "Zur Kenntniss der Spirochaete pallida," *Deutsche med. Wchnschr.*, Oct. 1905, 31, pp. 1665-67, 7 figs.
- UND HOFFMANN, E. "Vorläufiger Bericht über das Vorkommen von Spirochaeten in syphilitischen Krankheitsprodukten und bei Papillomen," *Arbeiten a. d. kaiserl. Gesundheitsamte*, 1905, 22, pp. 527-34, 2 figs.
- SERGEANT, EDM. ET ET. "Sur un flagellé nouveau de l'intestin des culex et des stegomyia, *Herpetomonas Algeriense*," "Sur un autre flagellé et sur des spirochaete de l'intestin des larves de moustiques," *Compt. rend. de la Soc. de Biol.*, 10 Fév., 1906, 60, pp. 90-93, 2 figs.
- SMITH, TH. "Grobe u. feine Spirillen im Darne eines Schweines," *Centralbl. f. Bakt.* (I), 1894, 16, p. 324.
- THEILER, A. "Spirillosis of Cattle," *Jour. of Comp. Pathol. and Therap.*, 1903, 47, p. 55; also, *ibid.*, 1904, 17; "Transmission and Inoculability of Spirillum Theileri (Laveran)," *Proc. Roy. Soc.*, Ser. B, 76, No. B512, pp. 504-6.
- TUNNICLIFF, R. "The Identity of Fusiform Bacilli and Spirilla," *Jour. Infect. Dis.*, 1906, 3, pp. 148-55, 1 plate.
- VINCENT, H. "Sur l'étiologie et sur lésions anatomo-pathologiques de la pourriture d'hôpital," *Ann. de l'Inst. Past.*, 1896, 10, p. 488, 1 plate.
- . *Recherches bactériologiques sur l'angine a bacilles fusiformes*," *Ann. de l'Inst. Past.*, 1899, 13, p. 609. 2 figs.
- WELLMAN, F. C. "Relapsing Fever: Its Occurrence in the Tropics and its Relation to 'Tick Fever' in Africa," *Amer. Med.*, 1905, 10, p. 151, 1 fig.
- ZETTNOW, E. "Geisseln bei Hühner- und Recurrens-Spirochäten," *Deutsche med. Wchnschr.*, March 8, 1906, 32, p. 376, 2 figs.
- ZIEMANN, H. Quoted by Laveran, 1903.

EXPLANATION OF PLATES.

The accompanying microphotographs are reproduced at a magnification of 1,500 diameters, with the exception of a few which are magnified 3,000 times and are so indicated. The preparations are stained by the Romanowsky method, unless otherwise stated.

PLATE 8.

Spirillum Obermeieri in blood of rat, showing divisional forms.

FIG. 1.—A group showing spirilla of different lengths. The two long ones above show transverse division.

FIG. 2.—The dividing form in the upper part of preceding figure, reproduced at a magnification of 3,000X. Note the clear space in the middle of the pale central zone.

FIG. 3.—A small group of spirilla. The longer one shows a pale central portion indicating transverse division. Note the fading away of the tips.

FIG. 4.—A dividing form at a magnification of 3,000X. Note the pale central portion and pale free ends.

FIG. 5.—A dividing form showing same features as preceding.

PLATE 9.

Sp. Obermeieri in blood of rat, showing agglutination forms.

FIG. 1.—An agglutination of two spirals which might be mistaken for longitudinal division. Note the pale tips and granular stains. Magnification, 3,000X.

FIG. 2.—An irregular agglutination or tangle of many spirals. Preparation stained by MacNeal's method (Bernthsen's insoluble methylene violet).

FIG. 3.—A long spiral thread the result of agglutination by means of the flagellum.

FIG. 4.—Agglutination of two cells by means of the flagellum. Note the delicate flagellum connecting the two cells. Löffler's flagellar stain.

FIG. 5.—Same as the preceding but with longer connecting flagellar bridge.

PLATE 10.

FIGS. 1-4 are of *Sp. Obermeieri* in blood of rat. They show a long free flagellum at one end and a rudimentary one at the other. Löffler's stain. The relatively greater thickness of the cells, as compared with those shown in the other photographs, is due to the intense staining.

FIG. 1.—Magnification, 3,000X. Note the pale appendage at end opposite from flagellum.

FIG. 2.—Flagellum as long as the cell.

FIG. 3.—Magnification, 3,000X. Note that the flagellum seems to be given off from the side of the tip.

FIG. 4.—Cell showing flagellum similar to that in Fig. 2; a marked stub at the other end.

FIG. 5.—*Sp. gallinarum*. Agglutination group in blood of chicken.

PLATE 11.

Showing phagocytic destruction of *Sp. Obermeieri* in the peritoneal cavity of an hyperimmunized rat. 6/132. MacNeal's stain. Mononuclear macrophages.

FIG. 1.—Free agglutination group of spirals; also phagocyte with fringe of spirals, some of which are almost completely absorbed.

FIG. 2.—Phagocyte with tangle of spirilla.

FIG. 3.—Phagocyte showing comma-shaped remnants of spirilla within its plasma.

PLATE 8.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.

PLATE 9.

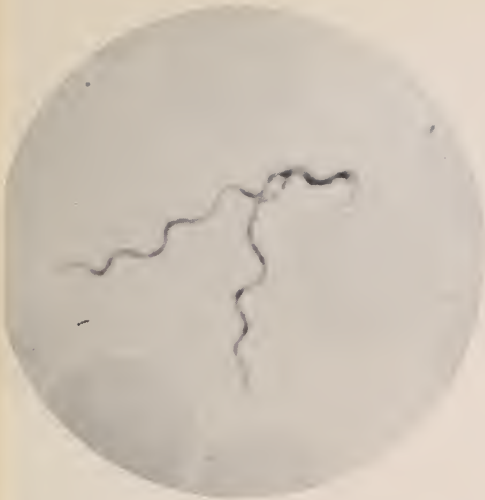


FIG. 1.



FIG. 2.

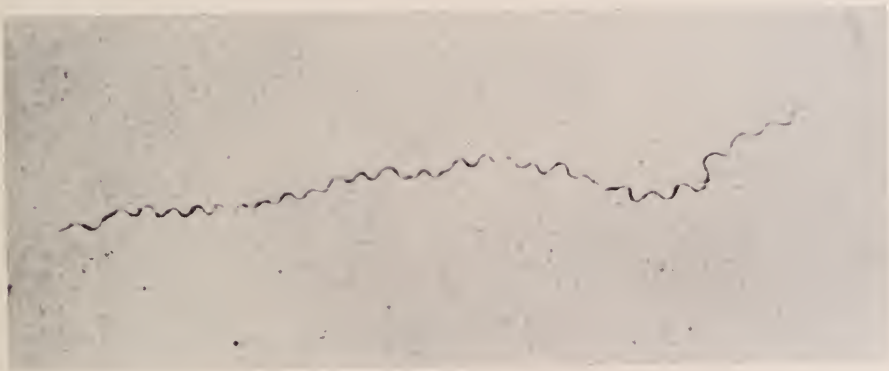


FIG. 3.



FIG. 4.

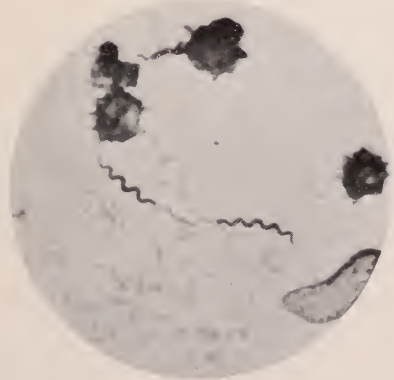


FIG. 5.



FIG. 1.

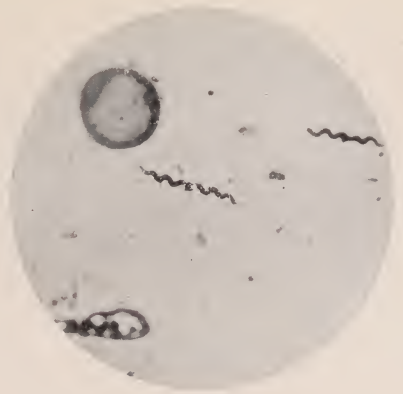


FIG. 2.

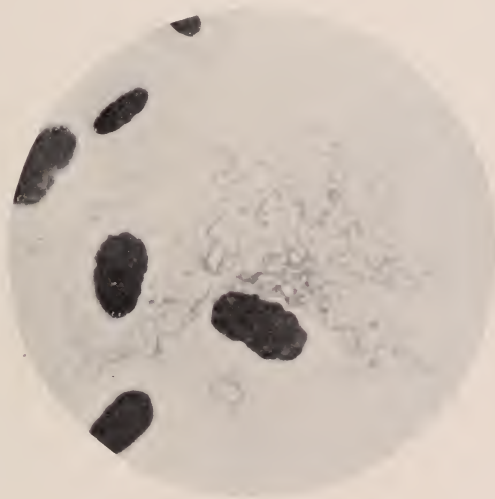


FIG. 5.



FIG. 3.

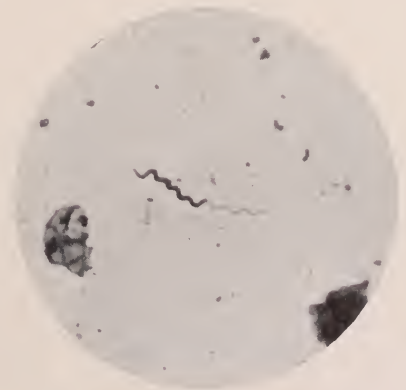


FIG. 4.

PLATE II.

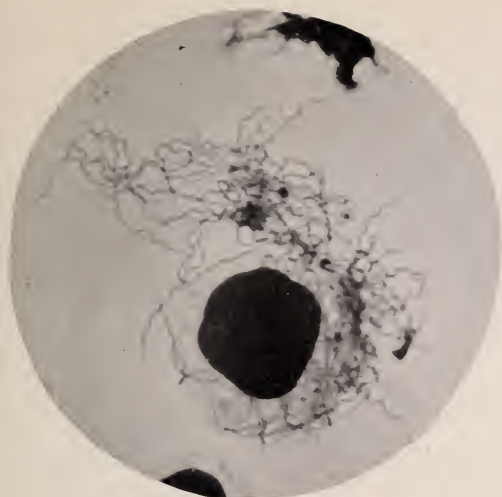


FIG. 1.

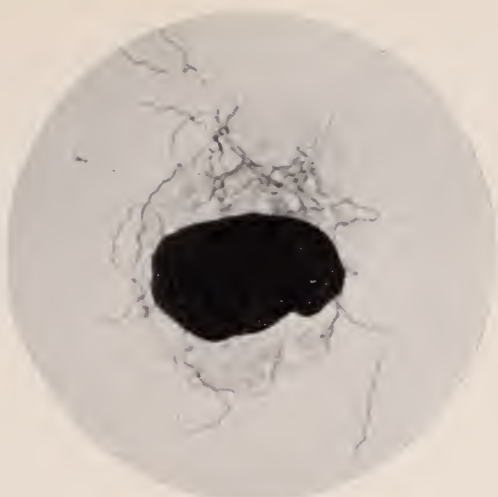


FIG. 3.

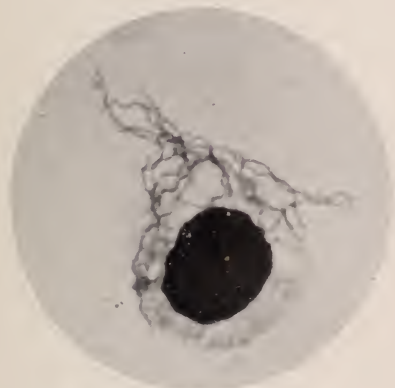


FIG. 2.

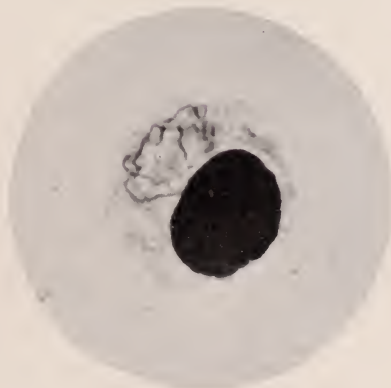


FIG. 4.

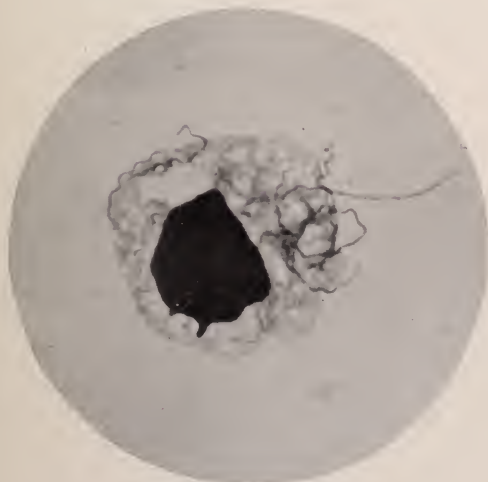


FIG. 5.

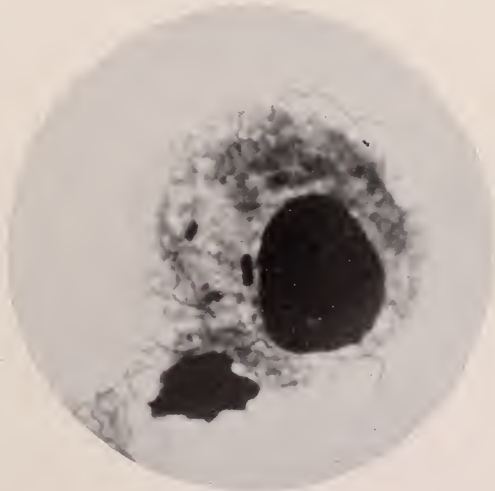


FIG. 6.

PLATE 12.



FIG. 2.



FIG. 3.



FIG. 1.



FIG. 4.



FIG. 5.

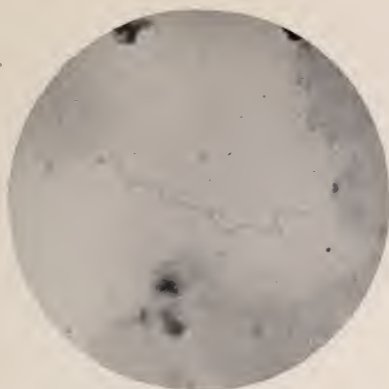


FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.

PLATE 14.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

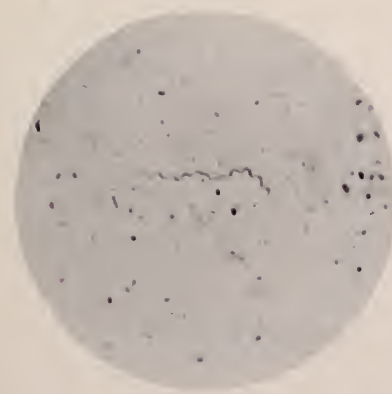


FIG. 5.

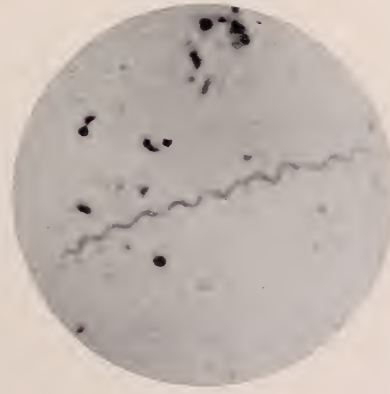


FIG. 6.

He was unable to infect *Glossinas* by feeding them on animals which contained trypanosomes in their blood. When fed on cattle, the blood of which was rich in trypanosomes, the flagellates disappeared from the stomach as the digestion of the blood progressed; there was neither a development of trypanosomes nor a permanent infection of the flies as a result. He attaches particular importance to the fact that some flies were infected when they fed on animals, such as oxen and mules, which had but a few trypanosomes in their blood, and he assumes, therefore, that not all of the blood trypanosomes are adapted for the infection of flies, but only such as occur in a definite, but as yet unknown, stage.

It follows from the foregoing that most, if not all, of the 60 infected flies referred to above contained trypanosomes at the time of their capture, and hence these were acquired while at large, and not as the result of experimental feeding. He first noted the presence of trypanosomes in the clear droplet which exudes when pressure is applied to the bulb at the base of the proboscis. The absence in the fluid of blood corpuscles and the presence of the trypanosomes in very large numbers, and in divisional stages, led him to conclude that the flies did not transmit the disease by carrying the blood directly from an animal to another, as is usually supposed, but that, as in the case of the malarial parasite, the trypanosomes pass through a development stage in the flies. This assumption he believed to be verified by the fact that on examining the flies he found that the stomach contained large quantities of trypanosomes in various stages of development, even when the stomach was entirely free from blood.

Among the tsetse trypanosomes he recognized two forms; one thick and plump, the other long and slender. The former, which stained blue, and had a rather large round nucleus of loose texture, he regards as the female; while the latter type, which does not stain blue, and has a long, narrow, rod-shaped, and very compact nucleus, he considers as the male cell. Whether the two forms conjugate directly, or whether the latter gives off microgametes, he leaves undetermined, though he inclines to the latter view.

In the lower part of the stomach he found forms which suggested fertilized female trypanosomes. These were large cells which con-

tained two, usually four, and at times eight, nuclei, although but one blepharoplast and one flagellum were present. This unusual condition of many nuclei and but one blepharoplast is certainly not the result of ordinary division where the division of the blepharoplast always precedes that of the nucleus.* He inclines to view it as a multiplication change consequent upon fertilization; the multinucleated trypanosomes eventually breaking up into many parts, thus giving rise to the youngest forms which first appear as round bodies with only a nucleus. Later these elongate to forms which have a nucleus and a blepharoplast, and these in turn develop a flagellum, and thus become true trypanosomes. Particularly important is the observation that rats injected with the fly trypanosomes failed to develop an infection.

In a second paper,¹ which appeared on the same date, Dr. Koch endeavors to establish differences between *Tr. Brucei* and *Tr. gambiense* based upon morphological variations of the trypanosomes found in *Glossina morsitans*, *Gl. fusca*, and *Gl. palpalis*, the first two flies being the carriers of *Tr. Brucei*, while the latter is that of *Tr. gambiense*, the cause of sleeping sickness. He points out, as in the preceding paper, that in the infected flies the trypanosomes grow much larger than in the blood of animals, and that they present the two types, female and male, referred to above. A comparison of the sexual forms of *Tr. Brucei* and *Tr. gambiense* showed differences which he thinks may be used to separate the organisms into two distinct kinds or species. The most important difference noted was the behavior of the blepharoplast in the female type. According to his statement the blepharoplast of *Tr. Brucei* is small, round, and 1.0μ in diameter; while that of *Tr. gambiense* stains intensely and is surprisingly large, $1.5 \times 2.5\mu$, is oval or rodlike, and always lies at right angles to the long axis of the trypanosome. A second point of differentiation—to which, however, he attaches less significance—is the size of the male types of both trypanosomes. For these he gives the following measurements:

*The occurrence of two or three nuclei with but one blepharoplast is not an uncommon feature in cultures of various trypanosomes. Thus, we have found such forms in cultures of *Tr. Lewisi*, *Crithidia fasciculata*, and in those of several bird trypanosomes.

¹"Über die Unterscheidung der Trypanosomenarten," *Sitzungsberichte der königl. preuss. Akademie der Wissenschaften*, Nov. 23, 1905, pp. 957-62.

		Length in μ	Width in μ
<i>Tr. Brucei</i>	} Female.....	25.0	3.60
<i>Tr. gambiense</i>		37.0	3.00
<i>Tr. Brucei</i>	} Male.....	40.2	2.10
<i>Tr. gambiense</i>		34.0	0.85

With reference to the above it should be noted that the trypanosome present in *Gl. palpalis* is assumed by Dr. Koch to be *Tr. gambiense*, while that in *Gl. fuscicauda* and *Gl. morsitans* was likewise assumed to be *Tr. Brucei*, notwithstanding no actual infection of flies by these organisms had been demonstrated, much less infection of animals by the bites of such flies, or by the injection of their stomach contents. Parenthetically it may be stated that a better and more certain means of differentiation is afforded, as we have repeatedly indicated, by the cultural characteristics of artificially grown, pure strains of trypanosomes. The marked differences presented by cultures of *Tr. Lewisi*, *Tr. Brucei*, *Tr. Evansi*, and of the various trypanosomes isolated from birds and mosquitoes, are sufficient evidence on this point, and it is somewhat surprising that the importance of the application of this method, so fundamental in bacteriology, does not seem to be recognized in protozoal work.

Having outlined at some length the observations made thus far on the trypanosomes of tsetse flies, it is in order to point out that, in our opinion, wholly erroneous conclusions have been drawn from the seen facts. It is our belief that the trypanosomes met with in the tsetse flies are "cultural" forms of harmless non-parasitic flagellates corresponding to the equally harmless *Herpetomonas* and *Crithidia* which we have found in mosquitoes. This view is based upon the following facts:

1. The large size of the trypanosomes as compared with that of the blood forms.
2. Their presence in flies which presumably had not fed on infected animals.
3. The failure to secure growths of the trypanosomes, pathogenic or non-pathogenic, in all, or even in a large per cent, of the flies fed on infected animals.
4. The failure to infect susceptible animals.
5. The analogy to mosquito trypanosomes.

Under No. 1.—It may be stated at the outset that an examination of the illustrations given by Gray and Tulloch, as well as those of Dr. Koch, suggests immediately that the trypanosomes in question are cultural types corresponding, in general, to the various forms which we have cultivated during the past three years. This view received definite confirmation during the subsequent study of the smears made from tsetse flies.

We have repeatedly called attention to the existence, in cultures of diverse trypanosomes, of two types quite unlike in many respects. A particularly good example of this duality of form is seen in the cultures of *Tr. avium* which is characterized by multiplication rosettes and free-swimming threadlike spirochetes. The term "spirochete" must no longer be employed to designate this form, since, unlike the true spirochete, it possesses the typical structure of a trypanosome; that is to say, it has a nucleus, blepharoplast, undulating membrane, and a whip, all of which are easily demonstrated. Inasmuch as corresponding forms were met with in the blood, the view was expressed¹ that the large form of blood trypanosome gave rise, in cultures, to the rosette type and that it represented the female cell, while the small form gave rise to the spirochete-like type which may be considered as an indifferent or asexual form.

As indicated above, Dr. Koch regards the wide type met with in tsetse flies as the female, and the long narrow type as the male trypanosome. In this respect, then, they present a marked "cultural" characteristic. Another feature which indicates that the tsetse trypanosomes are cultural forms is seen in the position of the blepharoplast which usually lies anterior to the nucleus, exceptionally by the side or posterior to it. Again, in cultural forms the ratio of the width to the length of the cell is invariably less than that of the blood forms. Thus, while the ratio of the small blood form of *Tr. avium* is about 5 to 20 μ , that of the corresponding spirochete-like type is 1 to 30–60 μ . In other words, the bulk or mass of the cultural trypanosome is always less than that of the blood form.

It may be taken as a general proposition that a given organism, bacterial or protozoal, when it undergoes rapid multiplication gives rise to smaller forms than it does under inverse conditions. And

¹ F. G. NOVY AND W. J. MACNEAL, "On the Trypanosomes of Birds," *Jour. Infec. Dis.*, 1905, 2 p. 287.

FIG. 4.—Phagocyte showing complete inclusion of several spirals.

FIG. 5.—Note the straight form of spiral showing transverse division. The phagocyte shows large numbers of comma or S-shaped remnants within its plasma.

FIG. 6.—Large phagocyte, the plasma of which is filled with chromatin particles; hence the dark portions in the plasma. The granules are probably the result of an enormous destruction of spirilla. Some comma-shaped remnants and three bacilli can be seen within the cell.

PLATE 12.

Spirillum Duttoni, or tick-fever spirillum from blood of a rat. Preparation of Dr. J. L. Todd.

FIG. 1.—Group of spirals. The long form shows a pale central portion indicative of transverse division. Note the pale free tips; the greater length of the spirals and the large, deep wavy bends, as compared with *Sp. Obermeieri* (Plate 8, Fig. 1, etc.); also note the tendency of the spirals to form circles and figure-8 forms.

FIG. 2.—A cell with typical large bends and pale tips.

FIG. 3.—A spiral showing clear central zone, evidently dividing.

FIG. 4.—One of the spirals here shown has three clear zones; division into vibrio form?

FIG. 5.—A spiral coiled in form of a circle; a very common condition.

PLATE 13.

Spirillum of relapsing fever from blood of man, Bombay case. Preparation obtained through Dr. W. S. Patton.

FIG. 1.—Perfect form with slight evidence of division into segments.

FIG. 2.—Very long form showing curled pale ends and some evidence of segmentation.

FIG. 3.—A spiral showing four S-shaped segments. The division in the middle gives two short spirals, which in turn divide into the S-form. 3,000X.

FIG. 4.—This shows a tendency to break up into comma-shaped segments. 3,000X.

FIG. 5.—Long spirillum resulting from the agglutination of three short or medium forms. Note the curling ends on account of which the cells do not coincide as perfectly as in Plate 9, Fig. 3.

FIG. 6.—An agglutination tangle. Note the tendency of the spirals to form loops or knots.

PLATE 14.

Spirillum glossinae, n. sp., and *Sp. pallidum*.

FIG. 1.—*Spirillum glossinae*, single cell; showing perfect spiral with faint tips. From *Glossina palpalis*, No. 8.

FIG. 2.—Two short spirals from the same preparation.

FIG. 3.—Two double length spirals from the same slide.

FIG. 4.—Three long spirals, like preceding. Note the clear space, showing transverse division, in the straightened out spiral.

FIG. 5.—*Treponema (Spirillum?) pallidum* from liver of a case of congenital syphilis. Preparation of Dr. F. Schaudinn.

FIG. 6.—Another specimen from the same preparation. Magnification, 3,000X. Note the faint tips and a light transverse zone in the middle.

THE TRYPANOSOMES OF TSETSE FLIES.*

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INFLUENCED by the studies of Schaudinn regarding the presence of trypanosomes in the gut of mosquitoes, many workers have endeavored to apply the same method of investigation to the solution of related problems. Thus, the trypanosomes and trypanoplasmas of fish have been traced to the flagellates in the intestine of the leech; the trypanosome of the rat to like flagellates present in the louse; and, more recently, *Tr. gambiense*, the cause of sleeping sickness, and *Tr. Brucei*, that of nagana, have been ascribed a direct relationship to the flagellates present in the stomach of the tsetse fly.

Inasmuch as all of our work during the past two years has shown that the results of Schaudinn are open to an entirely different conclusion from that arrived at by him, and quite generally accepted by others, it was of interest to examine into the supposed relationship of the two pathogenic trypanosomes mentioned above to the flagellated forms present in the stomach of the flies. The results obtained in our studies of the flagellates of mosquitoes† justified the belief that intestinal parasitism due to relatively harmless flagellates would be found to be a common natural condition.

In this examination we were not confined to a mere critical study of the published papers dealing with this subject, but through the kindness of Lieutenant A. C. H. Gray, R.A., M.C., member of the Sleeping Sickness Commission, we received a number of smears from six tsetse flies (*Glossina palpalis*) captured at Entebbe, Uganda, Central Africa. All of these contained trypanosomes, some in very large numbers, and consequently it was possible at first hand to make a microscopic and photographic study of the organisms. It is a pleasure, therefore, to acknowledge this very great courtesy of Lieutenant Gray, without whose aid it would have been quite impossible to arrive at definite conclusions.

* Received for publication March 14, 1906.

† Novv, MacNeal, and Torrey, "Mosquito Trypanosomes." An abstract of the paper read before the Society of American Bacteriologists, Dec. 28, 1905. Appeared in *Science*, Feb. 9, 1906, p. 207.

Before taking up the description of the trypanosomes present in the slides sent by Dr. Gray, it will be desirable to review at length the publications which have already been made on this subject. This is all the more necessary since a mere consideration of these papers will show how readily erroneous conclusions may be drawn from otherwise simple facts.

In order to ascertain whether the trypanosome of sleeping sickness could multiply in the stomach of *Glossina palpalis*, Gray and Tulloch¹ fed flies which had been caught 24 to 48 hours previously, on monkeys infected from the cerebrospinal fluid of sleeping-sickness cases. The flies were refed on infected monkeys every 48 hours.

An examination of the flies 96 and 120, and even as early as 24 hours after feeding showed that the blood in the intestines literally swarmed with trypanosomes. By successive feeding on blood the flies were found to maintain the trypanosomes in greatly increased numbers up to 12 days after the infective feed. It is significant that 10 per cent of all the flies, examined 24 hours after feeding, showed this multiplication of trypanosomes. The authors do not commit themselves to the extent of regarding these trypanosomes as actual multiplication forms of *Tr. gambiense*. They noted the very suspicious fact that on some days a considerable number of flies would be examined with negative results, although they were kept under the same conditions and fed on the same monkeys as at other times when successful results were obtained.

The appearance of the trypanosomes within 24 hours after feeding and the further important fact that many batches of flies failed to show any trypanosomes on identical feeding, led to an examination of freshly caught flies which were fed on an uninfected monkey. It was found that two out of 200 flies examined contained the same enormous numbers as those found in the flies which fed on infected monkeys. In other words, 1 per cent of the flies fed on uninfected monkeys, while 10 per cent of those fed on infected monkeys, showed trypanosomes. Since then infected flies have been met with more often, for the specimens which we have studied came from flies which had not even been fed on animals. In his letter Dr. Gray makes

¹ A. C. H. GRAY AND F. M. G. TULLOCH, "The Multiplication of the *Trypanosoma Gambiense* in the Alimentary Canal of *Glossina Palpalis*," *Reports of the Sleeping Sickness Commission, of the Royal Society*, No. VI, August, 1905, pp. 282-87. Four figures. Harrison & Sons, London.

the very significant statement that 8 per cent of the freshly caught flies from the uninhabited island of Kimmi contained these flagellates, while only 2 per cent of those taken in Entebbe were infected.

Extremely important as bearing upon the nature of the fly trypanosome is the fact that not one of five monkeys which received injections of enormous numbers of these flagellates developed an infection. The failure to produce an infection clearly indicates that the trypanosomes are not derived from the *Tr. gambiense* of the monkey's blood. The latter organism was never present in the blood of monkeys in larger number than one in six fields of the 2 mm. objective, and the negative inoculation experiments simply indicate that the *Tr. gambiense* which had been taken into the stomach of the fly had died off in the interval.

According to Gray and Tulloch, the trypanosomes seen in the fly vary from very small ones, some $20\ \mu$ in length, to very long slender ones of about $100\ \mu$. Attention was called to the position of the micronucleus, which was rarely seen at the extreme blunted end of the parasite. It was found to vary from a position midway between the posterior extremity of the trypanosome and the macronucleus, to a position on the anterior or flagellar side. They also noted the presence of divisional forms, as well as of rosettes, the members of which were joined directly by their posterior ends. These groups consisted of four to seven cells, or of large, loosely woven masses of 15 to 20 cells. The trypanosomes were found throughout the blood in the whole alimentary tract.

The trypanosomes of tsetse flies have further been studied by Dr. R. Koch,¹ who at first found them in *Glossina fusca*. Of 60 infected flies one was *Gl. morsitans*, and one was *Gl. pallidipes*, and for this reason he concludes that the tsetse fly disease in the German Protectorate is not exclusively carried by *Gl. morsitans*, but that it is also conveyed by *Gl. pallidipes* and chiefly by *Gl. fusca*. This conclusion, since it is based primarily upon the presence of flagellates in the flies, it will be seen is open to question. The percentage of infected flies is not given by him, except in the case of one locality, where the highest percentage (17.4) was observed.

¹ R. KOCH, "Vorläufige Mitteilungen über die Ergebnisse einer Forschungsreise nach Ostafrika," *Deutsche med. Wchnschr.*, Nov. 28, 1905, pp. 1865-69. 24 figures.

found to be present, and from this mixture slide preparations were made. The smears were stained by a modified Romanowsky-Nocht method, which will be described in a subsequent publication.

A study of these preparations showed: *first*, that we had to deal with what has already been designated as cultural forms, in distinction from what might be called the blood form of trypanosomes; *second*, that considerable diversity of type existed, sufficiently so as to justify the opinion that two, and possibly more, species of trypanosomes were present in the specimens examined. Our previous work with cultures of bird trypanosomes made it evident that two very unlike forms may still belong to the same species (as in the case of *Tr. avium*), and consequently, in studying the tsetse trypanosomes, this fact was taken into consideration at the outset.

The preparations from Fly No. 9 were particularly rich in trypanosomes which stained quite well, and were hence adapted for a careful examination. Apparently only one species of trypanosome was present in this fly, although under two very unlike forms. The difference, however, is no greater than in the case of *Tr. avium* already referred to. Moreover, these two forms correspond very well to the male and female types described by Dr. Koch. In honor of Dr. Gray, who first studied this species, it is here designated as

TRYPANOSOMA GRAYI, N. SP.

As mentioned, this species occurs in *Gl. palpalis* under two forms. The first of these corresponds to the free-swimming or spirochete-like form of *Tr. avium*, and bears considerable resemblance to the *Herpetomonas* of mosquitoes (Plate 15, Figs. 1 and 2). This, it will be seen, corresponds to the second or "male" type of Dr. Koch. The body, which is very slender and scarcely stains blue, measures 18-29 μ in length and from 0.6 to 1.0 μ in width. It is provided with a long, free whip, which is as long as, or longer than, the body proper. The total length, flagellum included, is from 36 to 48 μ . The anterior end of the body tapers out very gradually along the flagellum, and the existence of a rudimentary undulating membrane may be assumed. The flagellum terminates at, or near, the blepharoplast which is a conspicuous, deeply stained, perfectly round body lying immediately anterior to the nucleus and of about the

same diameter ($0.6\ \mu$) as the body of the cell. Immediately posterior to the blepharoplast is the very prominent, heavily stained, compact nucleus. This is markedly rod-shaped and measures from 2.3 to $3.3\ \mu$ in length by 0.5 to $0.6\ \mu$ in width. There is a faint indication of the presence of a diplosome, posterior to the nucleus. The posterior end is sharply pointed; the distance from the tip to the nucleus is from one and one-half to two times the length of the nucleus; i. e., 4.6 to $8\ \mu$. No observations have been made regarding the divisional forms of this type which was quite numerous in the preparations.

The second form corresponds in general to the "female" type described by Dr. Koch, though, as will be seen, the blepharoplast is much smaller than as stated by him. In the adult or full-grown stage it may be characterized as a wide and long, ribbon-like form. The plasma is granular, stains a deep blue, and may show colorless globules—a very common occurrence in cultural trypanosomes. The anterior end terminates in a very short flagellum, 2 to $6\ \mu$ long. The flagellum is continued along the side of the cell for about two-thirds of its length and forms the border or support of a prominent and very wavy undulating membrane; and finally terminates in the achromic zone surrounding the blepharoplast. The latter is usually immediately anterior to the nucleus, though at times it may be lateral or even posterior. In form it is a short oval about $0.4 \times 0.8\ \mu$ and is placed transversely. It should be noted that the form designated by Dr. Koch as that of *Tr. gambiense* has a blepharoplast which is $1.5 \times 2.5\ \mu$. In none of the preparations studied, which, as stated, are derived from *Gl. palpalis*, and therefore should contain *Tr. gambiense*, if the views hitherto published be correct, has anything been found which approaches this so unusually large blepharoplast. In fact, the dimensions given exceed those of the nuclei of our trypanosomes, as can be readily determined from the accompanying photographs.

The nucleus is somewhat round, about $2\ \mu$ in diameter, and does not stain as solidly as that of the type just described, but instead shows a granular structure. It lies at a distance from the blunt or square posterior end corresponding to about one-third the length of the cell. The pronounced square-cut end may possibly be due

to initial involution changes, since some forms have a sharp posterior end. A body, probably corresponding to the acrosome or diplosome which we have described in *Tr. Laverani* and in the mosquito *Herpetomonas*, may often be seen near the nucleus, usually between this and the blepharoplast (Plate 16, Figs. 3 and 5). This body is about $3\ \mu$ long and stains unevenly. Possibly it may correspond to the large body which Dr. Koch designates as a blepharoplast.

This type of trypanosome is subject to considerable variation in size. The largest individuals which we have seen are about $50\ \mu$ long by 2.0 – $2.5\ \mu$ wide. Smaller forms of one-half to one-third this length are also present. In such the free flagellum is relatively longer than in the full-sized individuals ($7\ \mu$). It may make up one-half or even more of the total body length. Very young forms are shown in Plate 15, Figs. 4 and 6.

Divisional forms of various size are common and, as with other trypanosomes, the division begins with the blepharoplast, thus giving rise to forms with two blepharoplasts, two flagella, and one nucleus (Plate 16, Figs. 1 and 2). In the more advanced stage the nucleus divides, and then the body proper. The division is very unequal, as has been shown by Gray and Tulloch (Plate 16, Fig. 1). Under exceptional conditions, which it is difficult to define, the division of the nucleus may precede that of the blepharoplast (Plate 15, Figs. 3 and 5). In such cases, according to Dr. Koch, the number of nuclei may be two, four, or eight. We have never noted more than two.

Degeneration forms are also met with (Plate 15, Fig. 5). As can be seen from the photograph, such cells present a swollen appearance, and may show two nuclei with but one blepharoplast. As the result of plasmolytic changes, the protoplasm is shrunken from the ends, and, moreover, becomes banded. It is possible that the consecutive division of the nucleus without that of the blepharoplast, as just mentioned, is the result of similar degenerative changes, rather than a fertilization of the "female" cell, as supposed by Dr. Koch.

OTHER TRYPANOSOMES IN GLOSSINA.

A study of the preparations from the other five flies showed that in only two (Nos. 7 and 8) did the form correspond sufficiently to

justify the belief that those flies contained the above organism. Both types were present; the narrow or "male" type being identical with that of No. 9. The wide, or "female," form presented slight differences, such as a more pointed posterior extremity and less marked or less wavy undulating membrane, but such variations may be expected from cultures of different age and from different media, as must necessarily obtain in each fly. The *Tr. Grayi* may therefore be looked upon as the most common form of trypanosome in the tsetse. It constitutes Type 1.

Type 2.—A slightly different type from the preceding was met with in Fly No. 10. The narrow or "male" form possessed a longer and wider body, and, in addition, the nucleus, instead of being long and cylindrical, was round or oval (Plate 17, Fig. 1). The more common, or "female," form varied in length from about 15 to 35, and even 50 μ . Its width rarely exceeded 1.5 μ . The nucleus, unlike that of *Tr. Grayi*, was elongated and oval in form, but its relative position in the cell was about the same. The blepharoplast was usually posterior to the nucleus, in varying position, either very near the tip or near the nucleus; at times it was anterior to the same. The flagellum extended close along the body, usually without any marked wavy bends, and terminated as a short free whip 3 to 4 μ long. The posterior end was usually very sharply pointed (Plate 17, Fig. 2).

Type 3.—This was present in Fly No. 6 in rather small numbers. It is markedly different from any of the other forms (Plate 17, Figs. 3 and 4). The "male" form is very much like that of *Tr. Grayi*, but apparently it is wider and has a much longer free whip. The latter is considerably longer than the body, which measures about 12 μ in length and 1.5 μ in width. The blepharoplast is anterior or lateral to the nucleus, which is oval or rod-shaped. The "female" forms which stained a deep blue were all short, the body being 12–15, or at most 20 μ long, and 0.5–2.0 μ wide. The free flagellum measured 5–15 μ . This form, it may be added, is the only one which might possibly be regarded as having some relation to *Tr. gambiense*.

Type 4.—Another type quite unlike the others was found in Fly No. 5. Notwithstanding a most careful search nothing similar

since the tsetse trypanosomes, notwithstanding their great numbers, which indicate rapid multiplication, are much larger than the *Tr. gambiense* of human or mammalian blood, it follows that the latter cannot be considered as the antecedents of the former. To illustrate this point it will be sufficient to compare the size of the two types. The *Tr. gambiense* as met with in the human blood usually measures about $20\ \mu$ in total length, and only exceptionally does it attain a length of 25 to $30\ \mu$. Its width is usually from 1.4 to $2.0\ \mu$; only the divisional forms being slightly wider. On the other hand, the predominating form in *Gl. palpalis* usually measures 40 to $50\ \mu$ in length, and may even be longer, for Gray and Tulloch mention some as being about $100\ \mu$ in length. The width of these forms is usually about $2\ \mu$, but may at times attain $3\ \mu$. In other words the bulk or mass of the tsetse trypanosome is at least twice that of the blood form of *Tr. gambiense*. This we consider to be important evidence showing that the tsetse trypanosome is not derived from the *Tr. gambiense*, or for that matter from *Tr. Brucei*.

Another feature to which attention should be called is the enormously large blepharoplast which Dr. Koch describes in connection with the "female" type of *Tr. gambiense* as observed in the tsetse fly. Such a large structure has never before been seen in a pathogenic protozoon, but, without questioning the statement, it may be asked whether it is possible to get so enormous a structure in the course of a rapid multiplication of the blood trypanosome in which the blepharoplast is very small and measures only about $0.7\ \mu$. On *a priori* grounds one would expect under these conditions not only trypanosomes which are smaller than the blood forms, but also smaller blepharoplasts than such as are found in the blood forms. In our material, derived from *Gl. palpalis*, no trypanosomes could be found with blepharoplasts approaching in size that given by Dr. Koch.

Under No. 2.—The presence of trypanosomes in large numbers in tsetses which presumably have never fed upon animals is a fact which argues strongly against a possible relationship to *Tr. gambiense* or *Tr. Brucei*. We are informed by Dr. Gray that a greater proportion of the flies examined contained no traces of blood in their intestinal tracts, and this also seems to be the case in Dr. Koch's

observations. It is very significant, as Dr. Gray writes, that flies captured on the uninhabited island of Kimmi should show 8 per cent infected, whereas those taken along the shore at Entebbe, where there is an abundant opportunity for infection from man, should show only 2 per cent of infections. Dr. Koch's exceptional observation of 17.4 per cent of infected tsetse flies in Luengeratal, where few antelopes, but many sheep and goats, were present, is hardly explainable by the supposition that they are derived from the latter. It would be well to study the stomach trypanosomes of tsetse flies in regions where trypanosomiasis is rare or absent.

Under No. 3.—As pointed out above, Gray and Tulloch repeatedly met with batches of flies which, after feeding on infected monkeys, failed to show trypanosomes, although other lots fed on the same animals, under identical conditions, gave a small number of positive findings. Their total of 10 per cent of infection in flies which were thus fed is insufficient evidence to show that they are derived from the trypanosomes in the blood of the monkeys. The further fact that the monkeys contained but very few trypanosomes, about one per six fields, whereas the flies examined 24 hours after feeding contained enormous numbers, is not explainable on the supposition that these have resulted from the multiplication of the scanty parasites present in the blood which the flies had sucked up. The time was altogether too short for such multiplication to have taken place. On the contrary, it is evidence that shows that the flies were infected at the time of their capture. The fact that Dr. Koch failed to find the trypanosomes in tsetse flies which fed on richly infected cattle, and obtained but few trypanosomes in flies which fed on oxen and mules, goes simply to show that the mammalian trypanosomes do not multiply in the tsetse flies. It does not necessitate recourse to the assumption of the existence of some unknown stage of the mammalian trypanosome which is especially adapted or necessary for multiplication in the fly.

Under No. 4.—The failure to infect monkeys by injecting enormous numbers of tsetse trypanosomes, as noted by Gray and Tulloch, and the similar failure of Dr. Koch to infect rats, may be taken as crucial evidence of the non-identity of those flagellates with *Tr. gambiense* and *Tr. Brucei*. Actively growing cultures of *Tr. Brucei*

and *Tr. Lewisi* almost invariably infect when injected into animals and a like result should be obtained if the tsetse trypanosomes, which are present in large number and in active multiplication in the fly, have any relation to the pathogenic forms.

The negative experiments referred to cannot be explained satisfactorily in any other way than that the pathogenic trypanosomes which were sucked up died out in a short time in the stomach of the flies. In mosquitoes which are allowed to feed upon a rat infected with *Tr. Brucei*, the latter organism may be seen alive for 24 to 36 hours and as long as it is alive such contents will infect mice and rats. After this period, *Tr. Brucei* cannot be found in the contents, and infection cannot be brought about in animals, notwithstanding the presence of flagellates which, as shown in the previous paper, are the result of a natural infection.

Under No. 5.—The presence of flagellates in the stomachs of tsetses presents a striking analogy to the presence of like forms in the gut of the mosquito. In the case of the latter we have conclusively shown that 15 per cent of the captured or "wild" mosquitoes have a flagellate infection with *Herpetomonas*, *Crithidia*, and possibly other species. It would obviously be false logic if we were to examine mosquitoes, after allowing them to feed on rats infected with *Tr. Lewisi* or *Tr. Brucei*, and finding flagellates present were to declare that they were multiplication forms of these two well-known species of trypanosomes. And yet such a conclusion would be fully as justifiable as the assumption that the forms met with in the tsetse were derived from either *Tr. Brucei* or *Tr. gambiense*. There is every reason to believe that not one, but several species of trypanosomes, in no wise related to the pathogenic forms, may find their natural habitat in the stomach of the tsetse flies. This is certainly true for the mosquito, and it is not far-fetched to hold that the latter is not the only one in this regard.

The question naturally arises: If the trypanosome in *Gl. palpalis* is not *Tr. gambiense* and that in *Gl. fusca* and *Gl. morsitans* is not *Tr. Brucei*, what are they then? No answer to this can be given other than that they are probably harmless non-pathogenic forms. That they are "cultural" forms, there can be no question, and in view of the success of cultivating the mosquito flagellates,

it is quite certain that the tsetse trypanosome can be readily cultivated *in vitro*. Such cultures may be expected to show identically the same form as that seen in the fly, and, moreover, such cultures, as seen from the experiments with flies, will be found to have no action on ordinary experimental animals.

As to the origin of these flagellates we can only say that we know as yet but too little of the distribution of trypanosomes in nature to arrive at any definite supposition. If it is assumed that the infected mosquitoes (15 per cent) and infected tsetses (2-17 per cent) derive their parasites from the blood of animals, it follows that a much larger per cent of these insects must feed, at least once, on animal blood. In other words, there are at least as many uninfected animals bitten, as those which are infected and from which the insects supposedly derive their infection. This would mean that more than 30 per cent of the biting insects, as they live in nature, feed at least once on the blood of some animal. It would seem, on *a priori* grounds, that this number is altogether excessive, and yet no proof to the contrary can be offered. Notwithstanding this, it is almost as reasonable to believe that only a very small percentage of biting insects ever feed on blood; and that such trypanosomes as they contain are derived from plant juices, stagnant waters, etc. The objection that flagellates have never been observed in such fluids is not a serious one. A careful search may yet reveal the parent form of the trypanosomes which are present in the alimentary tract of ordinary flies, tsetses, mosquitoes, and even in various larval forms. Their presence in the larvæ of mosquitoes, in butterflies, etc., certainly indicates that insects may develop a flagellate infection of the alimentary tract without ever having fed on the blood of infected animals.

THE TRYPANOSOMES OF *GLOSSINA PALPALIS*.

As indicated in the beginning, it was possible through the kindness of Dr. Gray to stain and examine smears from the intestinal contents of six tsetse flies (*Gl. palpalis*). These flies were all caught on the lake shore at Entebbe, and after being brought to the laboratory were at once dissected. A little fresh blood serum was added to the intestinal contents of those flies in which trypanosomes were

to the "male" forms described in connection with the other types could be found. Instead two very different forms were present. One of these stained a very light blue, and its body tapered along the flagellum almost to its tip. This form usually measured 40-50 μ in length and 1.5-2.0 μ in width. Although the body was wavy and snakelike, the undulating membrane did not show the wavy folds as in Type 1. The nucleus was cylindrical, as in Type 2, but unlike that of the latter, it was quite near, within 3 μ , of the posterior end, which was usually truncated, though at times it terminated in a sharp point. The oval blepharoplast was anterior to the nucleus.

This form, it should be noted, corresponds to the measurements which Dr. Koch gives for the male form of *Tr. Brucei*.

Associated with this form was another which stained a deep blue and had a very granular plasma. Its total length measures from 33 to 45 μ . Its widest part (3 μ) is about 6 μ from the posterior end, which tapers to a blunt point. Anteriorly the body constricts rather suddenly and becomes gradually narrower until it reaches the tip of the flagellum. At times a short free whip is present. The nucleus is roundish or oval and is made up of loose chromatin particles. The blepharoplast may be either anterior or posterior to the nucleus (Plate 17, Fig. 5).

Agglutination groups of the narrow form were met with, and in these the whips were directed outward. Occasionally two cells were found agglutinated, the posterior ends overlapping in exactly the same manner, as we have shown in our photographs of *Tr. avium*.¹ Divisional forms corresponding closely to those of Type 1 (Plate 16, Fig. 1) were present. Degenerative forms, as seen from the rounding up of the posterior end, were quite common.

In separating the forms observed into the several types described above it is not intended to convey the impression that these are distinct species. Whether they are distinct species or mere variations of one species cannot be settled at present by mere microscopic examination. Considerable variation in form is met with in a few of the cultures of trypanosomes which we have studied. This is notably true of *Crithidia*, which, while it usually appears in the

¹See *Jour. Infect. Dis.*, 1905, 2, Plate 10, Figs. 3 and 4.

typical short, truncated form, at times, under certain conditions of the medium or temperature, gives rise to long, spindle-shaped cells which could easily be supposed to represent a distinct species. Hence, extreme care must be exercised in interpreting the forms observed in the alimentary tract of insects. The conditions in no two insects are necessarily alike, and the kind and amount of food recently taken is not without influence on the size and number of the organisms present.

The purpose in presenting these observations is to call attention to what we have designated as the cultural characteristics of trypanosomes. The presence of such forms in the stomach or intestines means that these organisms can be cultivated artificially, and, that being the case, it is incumbent upon every investigator engaged in studying the living forms to obtain pure cultures of the organisms in question before drawing conclusions as to their supposed relationship to pathogenic trypanosomes or to intracellular parasites. As in bacteriological work, where the essential requirement of pure cultures is demanded, so also in this class of protozoal work the necessity of pure cultures can no longer be ignored. In our opinion, too many hasty conclusions have been drawn already from the mere morphological study of the flagellates observed in sanguivora, such as leeches, lice, mosquitoes, and flies.

The conclusion, in brief, to be drawn from this study is that the trypanosomes of the tsetse flies have nothing to do with the pathogenic *Tr. gambiense* or *Tr. Brucei*, and that they are mere harmless parasites of the alimentary tract analogous to those known to exist in mosquitoes.

As this paper is going to press our attention has been called to the results of a similar investigation carried out by Professor E. A. Minchin. The original is not accessible, but from an abstract it appears that he regards the tsetse fly as a mere carrier and not as a host of the trypanosome of sleeping sickness, and the flagellates which are present in its stomach he considers as parasite peculiar to the fly and in no wise related to the mammalian trypanosomes.

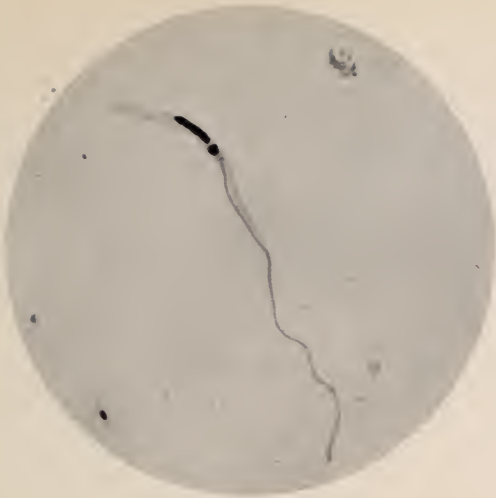


FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.

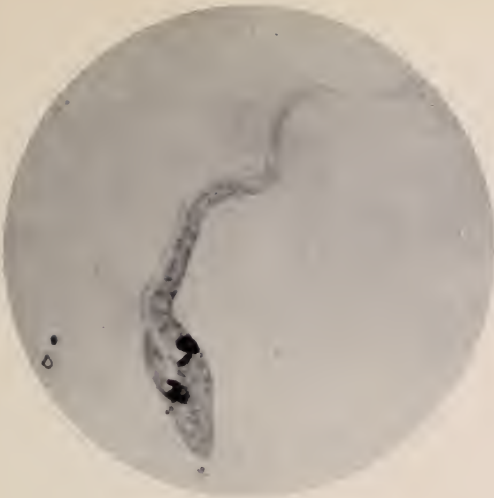


FIG. 1.



FIG. 2.

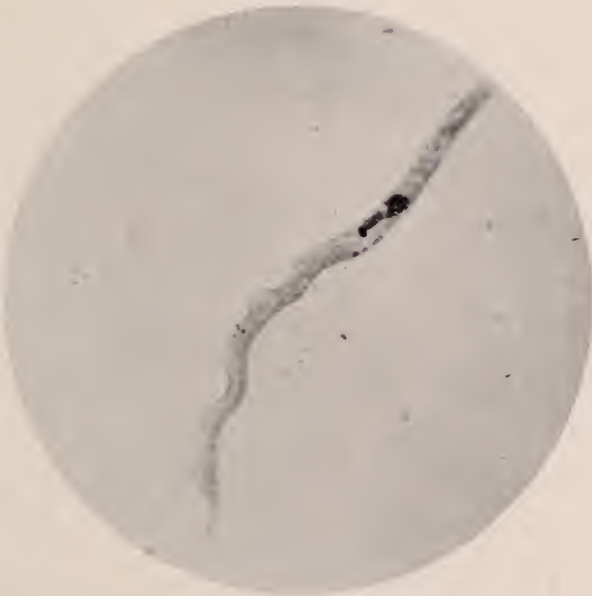


FIG. 5.

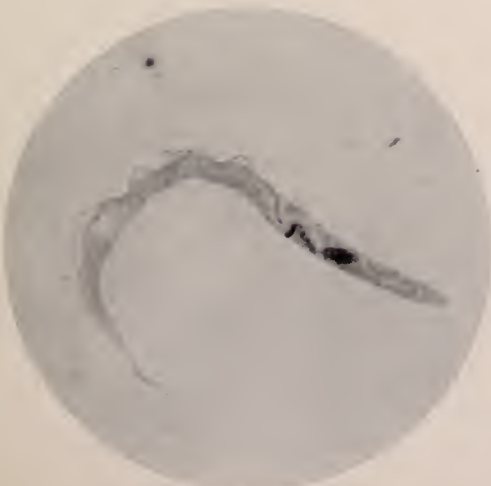


FIG. 3.



FIG. 4.



FIG. 1.

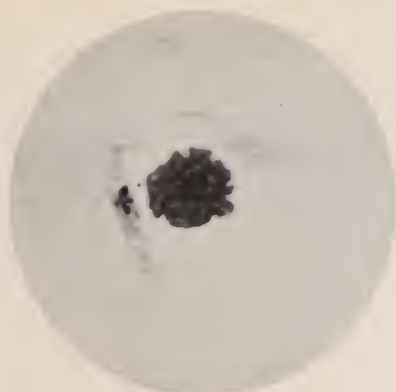


FIG. 3.

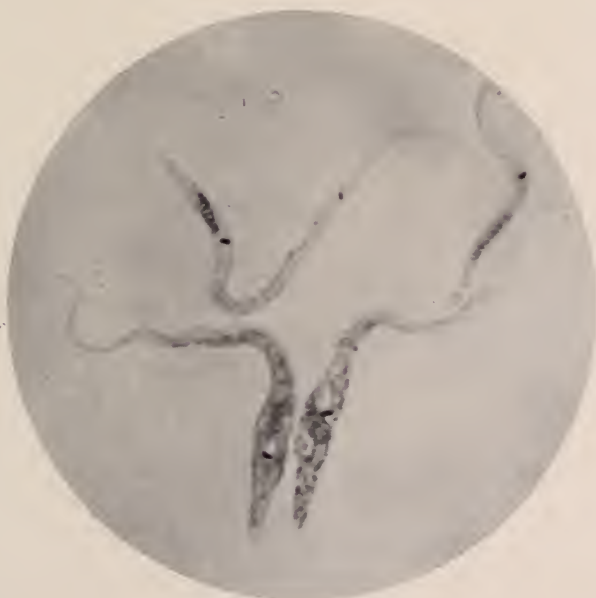


FIG. 5.

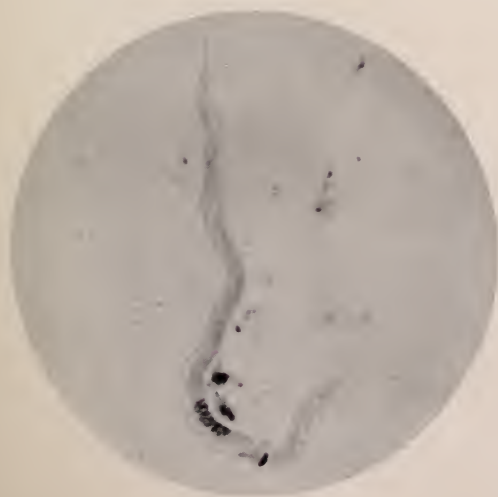


FIG. 2.

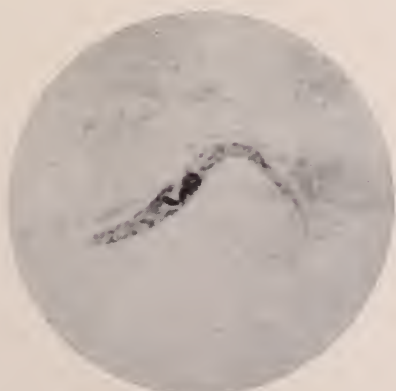


FIG. 4.

EXPLANATION OF PLATES.

The accompanying photographs are all taken at the uniform magnification of 1,500 diameters, and, as such, they are directly comparable with those of other cultural trypanosomes previously given.

PLATE 15. *TRYPANOSOMA GRAYI*, N. SP.

All the photographs are taken from Tsetse No. 9, except Fig. 4, which is from Fly No. 7.

FIG. 1.—A long, narrow, or "male" form. Note the free flagellum as long as the body; also the long cylindrical compact nucleus and the prominent round blepharoplast.

FIG. 2.—A similar form from the same preparation. Indication of a diplosome posterior to the nucleus.

FIG. 3.—A broad, blue-staining, or "female" form. Shows the nucleus in process of division and anterior to the oval blepharoplast; also the square posterior end.

FIG. 4.—A young form of the same type.

FIG. 5.—A degeneration of the same. Note the increase in width, also presence of two nuclei with but one blepharoplast. The darker portion, in parts broken up into bands, represents the blue-stained plasma which shows plasmolytic changes with consequent retraction from the cell-wall.

FIG. 6.—A very young form with the square posterior end of a very large trypanosome projecting into the field.

PLATE 16. *TRYPANOSOMA GRAYI*.

Figs. 1 and 2 are from Fly No. 7, the others are from No. 9.

FIG. 1.—A large "female" form in process of division; it shows two nuclei and two blepharoplasts. Note the unequal longitudinal division.

FIG. 2.—A smaller form, showing earlier stage of division, one nucleus and two blepharoplasts.

FIG. 3.—A very long form, showing the very prominent undulating membrane and the short free whip. Note the presence of the diplosome between the nucleus and the blepharoplast.

FIG. 4.—A young form of the same with a relatively long free whip.

FIG. 5.—A very long form, showing the diplosome between the nucleus and the blepharoplast; also prominent undulating membrane and short free whip; truncated posterior end.

PLATE 17. SHOWING TYPES 2, 3, AND 4.

FIG. 1.—A slender "male" form of Type 2 in Fly No. 10. Note the thick oblong nucleus and anterior to this the large round blepharoplast.

FIG. 2.—A long form of the blue-stained, or "female," cell from the same preparation as the preceding. Note the cylindrical, granular nucleus, and posterior to this the rod-shaped, transversely placed, blepharoplast; also the very short free whip.

FIG. 3.—A slender "male" form of Type 3 from Fly No. 6. Note the very long free flagellum.

FIG. 4.—The usual form of the blue-stained, or "female," of Type 3 from the same preparation as the preceding. The free flagellum can scarcely be seen.

FIG. 5.—The two forms of Type 4 from Fly No. 5. Below are two deeply blue-stained, highly granular, or "female," forms. Above can be seen the faintly stained, narrow form, with cylindrical nucleus lying close to the posterior end.

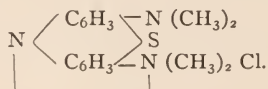
METHYLENE VIOLET AND METHYLENE AZURE.*

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THE more recent advances in the knowledge of protozoal diseases have been associated with the development of methods of study, especially differential staining methods, and of these the Romanowsky stain deserves primary consideration. This stain, as is well known, depends upon the combination of eosin with altered methylene blue. There is, however, a lack of definite knowledge concerning it, as is at once apparent from the large number of empirical modifications offered in the literature. Certain of these modifications have attained a wide employment as general stains for histological examinations of blood. For these reasons it has seemed worth while to undertake a critical examination of our present knowledge of these stains and to advance by experimentation to more definite proof the various explanations which have been offered concerning the chemical composition of the ripened methylene blue solution and its mode of action.

Our more exact knowledge of methylene blue and its derivatives dates from the observations of Bernthsen,¹ who determined the composition of methylene blue chloride as $C_{16}H_{18}N_3S\text{Cl}$, and assigned the following structure to it,

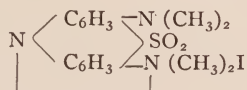


By treating methylene blue with dilute alkalis he obtained a mixture of bodies among which he recognized methylene violet, methylene azure, their leukobases and leukomethylene blue. He was able to prepare pure methylene violet as follows. Twenty grams of recrystallized methylene blue iodide and four liters of water were placed in each of three flasks, warmed, filtered, and freshly precipitated silver oxide (made from 17 grams of silver nitrate) added to the warm filtrate from each flask, thoroughly shaken, and again filtered. The three solutions were then combined and diluted to 14 liters, and silver oxide, prepared from 25 grams silver nitrate, added, and again thoroughly shaken and filtered. The iodine-free filtrate was heated in several large flasks and kept boiling for one-half to one and a half days. The solution was very early filled with green shining crystals of mythylene violet while the vapor contained dimethylamine, which was collected and identified by its chemical reactions. The mother liquid at the end of the reaction contained only a little methylene violet, but considerable methylene azure, in solution. Leukobases were not recognized.

* Received for publication April 9, 1906.

The precipitated methylene violet was filtered out and purified by repeated crystallization from alcohol, finally obtained as green, shining, needle-shaped crystals, almost insoluble in water, soluble in alcohol with a violet color and red fluorescence, in ether with a raspberry red color. It dissolved in warm dilute hydrochloric acid and reprecipitated on cooling as long dark needles. In concentrated sulphuric acid it dissolved with a violet blue color. These last two reactions are useful tests, the crystalline hydrochloride being obtained only with fairly pure methylene violet. Analysis indicated the formula $C_{14}H_{12}N_2SO$ which would be expected from its origin from methylene blue base by splitting off dimethylamine.

Methylene azure remained in the mother liquid after the precipitation of methylene violet by the prolonged boiling, together with a small amount of methylene violet. Bernthsen reduced the hot solution by means of stannous chloride and strong alkali. A half volume of alcohol was then added and the solution allowed to cool in a hydrogen atmosphere. The leuko-azure, crystallized out for the most part during the cooling, was filtered off in hydrogen atmosphere, washed in dilute alkaline stannous chloride solution and finally with pure water. It was then dissolved in dilute hydrochloride acid, oxidized by ferric chloride and salted out as a brown, amorphous precipitate containing some needle-shaped crystals. The chloride could not be purified, so potassium iodide was added to the solution and the iodide obtained in fine, green, shining needles and this repeatedly crystallized from hot water. The analysis indicated the formula $C_{16}H_{18}N_3SiO_2$, methylene blue plus two atoms of oxygen, with the following structure:



He was able to isolate methylene azure also by reduction of the solution as above and extraction with ether, carefully avoiding oxidation. The substance showed the following characteristics: its iodide was more soluble in water than methylene blue iodide, and fixed alkalis at once precipitated the free base and completely changed the color from blue to red, distinction from methylene blue; the chloride dissolved in concentrated sulphuric acid with green color, distinction from methylene violet.

Simon,² a student of Bernthsen who was engaged in this work, obtained results in the analysis of methylene azure indicating a distinctly different formula from the one finally selected by his teacher. He expressed himself as doubtful as to the purity of their methylene azure believing that it might still contain methylene violet. Some of the analyses upon which Bernthsen bases his formula were made by Simon.

Kehrmann and Schaposchnikoff³ have shown that in methylene-blue salts the sulphur atom may be tetravalent and the linking of the orthochinon type instead of the parachinon of Bernthsen. Unfortunately these workers do not appear to have investigated methylene violet and methylene azure.

After this brief review of the purely chemical investigation of the action of alkalis on methylene blue, it is necessary to consider the researches of those more particularly concerned with the histological application of these dyes. The pioneers in this work were Romanowsky and Unna.

Romanowsky⁴ in 1891 described a method of staining the hitherto unstained portion of malarial parasites by means of a mixture of aqueous solutions of methylene blue and eosin. The part of the parasite in question was stained in a carmin violet

color and recognized as the nucleus by Romanowsky. The staining was produced by adding an aqueous solution of eosin to an aqueous solution of methylene blue until a precipitate began to form. Simultaneously with the formation of this precipitate a new dye had been produced which had the elective affinity for the chromatin of the plasmodium. A slight excess of eosin or of methylene blue in the mixture was fatal to the nuclear stain. The exact ratio proved different with different brands of methylene blue, and even in the same solution it changed with age. Old methylene blue solutions covered with scum seemed to work best.

In the same year Unna⁵ obtained his first red-stained mastzellen by accident when he was using an old solution of the composition,

Methylene blue	1.00 g.
Potassium hydroxide	0.05
Distilled water	100.00 c.c.

With a fresh solution he could not duplicate the result. There had been here a ripening. This ripening was not due to bacteria; hydrogen peroxide and carbonic acid failed to alter the fresh solution so that it would give such a stain. Therefore he concluded that the change must be due to the alkali. An old alkaline solution shaken with ether was found to color the latter an intense red (methylene violet). A solution rich in methylene violet was then made as follows:

Methylene blue	1 g.
Potassium carbonate	1
Alcohol	20 c.c.
Distilled water	100

Boil slowly on the water-bath until 100 c.c. remain. A dark violet solution resulted which has become widely known as Unna's polychrome methylene blue.

Ziemann⁶ showed that certain brands of methylene blue could be used for Romanowsky staining when freshly made up, while others were useless for this purpose.

Nocht⁷ conceived the idea that the nuclear stain might be due to some other dye present as an impurity in methylene blue, becoming active only when the eosin and methylene blue had precipitated each other. So he added some of Unna's polychrome methylene blue to the Romanowsky staining mixture and found that the red nuclear stain was improved. With the polychrome methylene blue and eosin alone he was unable to get any result until the alkalinity of the former had been considerably reduced by the addition of dilute acetic acid. Then the chromatin red was produced, but cytoplasm was not well stained until methylene blue was again added to the mixture. A mixture of neutralized polychrome blue, methylene blue, and eosin in water stained in a few hours, but did not overstain in 24 hours. Nuclei of the older forms of malarial parasites were not stained by this procedure. In a later note⁸ he reported that if methylene blue solution be made weakly alkaline, heated a few hours in a steamer, filtered, neutralized, and added to unaltered methylene blue, the final mixture, having a clear blue color, would contain the essential nuclear stain; thus the use of Unna's polychrome methylene blue was dispensed with.

Nocht⁹ subsequently showed that a new dye was developed in the ripened methylene blue, by extracting the solution with chloroform, which took on a red color. Evaporation of the chloroform at room temperature left a residue, "*roth aus methylene blau*," soluble in cold water with red violet color and neutral reaction. This *roth aus methylene blau* alone, in some experiments, stained the nuclei of young forms of malarial parasites, but not of the older forms. The same proved true of a solution

of *roth aus methylene blau* in methylene blue and of a mixture of *roth aus methylene blau* with eosin. When, however, all three dyes, methylene blue, *roth aus methylene blau*, and eosin were brought together, the nuclei of the parasites stained without exception a brilliant red, even in the crescent forms.

Alkaline solutions were found to stain more readily than neutral ones. He considered Unna's polychrome blue essentially a solution of *roth aus methylene blau* in methylene blue. Its alkalinity was found to be variable and Nocht emphasized, therefore, that it should be neutralized, always, in using it for Romanowsky staining. However, he no longer recommended this solution as he has prepared a better one by heating a solution containing 1 per cent methylene blue and $\frac{1}{2}$ per cent sodium carbonate at 50° to 60° C. for two days. At the end of this time the solution is still pure dark blue in color, but contains sufficient *roth aus methylene blau* to give excellent chromatin staining. It is used by adding two or three drops of 1 per cent aqueous eosin to 2 c.c. of water and then adding the ripened blue solution slowly until the mixture becomes so dark that the eosin color is hardly to be recognized. Float the preparation five to ten minutes. If at the end of this time a second preparation be put on the same mixture, the specific chromatin stain no longer appears, or only faintly after a long time. Therefore the mixture should be used but once.

Concerning the chemical identity of *roth aus methylene blau*, Nocht could only say that it was not identical with either methylene red or methylene violet.

Other methods of developing the red nuclear stain have been devised, such as the adding of silver oxide or borax to methylene blue, but they all depend upon the principle of Nocht, that is, to set free the methylene-blue base and allow it to decompose, the process being hastened by heat in most instances. Slight modifications of Nocht's method have been widely used with good success.

Louis Jenner¹⁰ made a compound of methylene blue and eosin by mixing alcoholic or aqueous solutions of these dyes. This substance he was able to prepare quite pure with melting-point of 227° , and he regards it as a linking of methylene blue (base) and eosin (acid). By dissolving it in methyl alcohol he made an excellent fixing and staining reagent for blood smears. His stain does not, however, contain the nuclear staining principle of Romanowsky, but his work bears an important relation to this subject, chiefly because he introduced this valuable way of using methyl alcohol, which was later adopted by Leishman.

Michaelis¹¹ first undertook to explain the chemical nature of the Romanowsky stain and gives a quite complete summary of Bernthsen's work bearing upon it. His own experiments are not detailed, but he has concluded that methylene azure and methylene violet are produced by the decomposition of methylene-blue base, in different relative quantities according to the conditions of the experiment. By the rapid oxidation of methylene-blue base (silver oxide method) methylene violet is formed in excess; but in the gradual decomposition of methylene-blue solution treated with alkalis, methylene azure is the chief product.

As a distinguishing test he gives the reaction with concentrated sulphuric acid. Methylene blue dissolves in this acid with a green color, in dilute sulphuric or hydrochloric acid with a blue color. Methylene azure also dissolves in concentrated sulphuric acid with green color. Methylene violet, on the other hand, never shows the green color but gives a blue color with the acid. The test is made by bringing a dilute solution of the dye into a test tube containing the acid (ring test). Through Ehrlich he obtained samples of methylene violet and methylene azure. With the former he

was unable to make stained preparations for the free base was so insoluble that, by a saturated aqueous solution, nuclei were only slightly tinted blue. The hydrochloride likewise proved useless. Methylene azure (hydriodide), on the other hand, stained nuclei in tissue sections an intense blue, mucous and mastzellen granules red. Upon testing Unna's polychrome methylene blue with concentrated sulphuric acid he obtained a green ring, which indicated that methylene violet was not present in great amount as he had expected on account of its almost absolute insolubility in water. Methylene *roth* is absent, as it is destroyed by alkalis, by the action of which Unna's polychrome methylene blue is produced. The green ring with sulphuric acid indicates that the dye is chiefly methylene azure. One might suppose that this green ring is produced by undecomposed methylene blue, but he diluted the polychrome methylene blue with water, added sodium hydroxide, extracted with ether, and found that the water became colorless below the ether, from which he concluded that no trace of methylene blue was present. Michaelis regards azure as the essential nuclear dye and was able to stain nuclei red with it alone, that is, without the presence of eosin. For staining he recommended the following (slightly modified Nocht's) solution which is for sale by Grüber under the name of *azur-blau*. Dissolve 2 grams of methylene blue in 200 c.c. of distilled water, add 10 c.c. of tenth normal sodium hydroxide and boil 15 minutes, cool, and add exactly 10 c.c. tenth normal sulphuric acid, filter. To use add one part of this solution to five parts of aqueous eosin solution, mix and stain the fixed preparation 15 minutes. It stains only where the smear is thin.

Reuter¹² and Leishman¹⁴ independently conceived the idea of utilizing the precipitate formed by mixing Nocht's solution with eosin. Reuter¹³ examined the ethereal extract of Nocht's solution (dry Nocht's *roth aus methylene blau*) and found that when dissolved in water and mixed with eosin it gave rise to no chromatin staining. When a few drops of acetic acid were added to the extract, it formed a precipitate with eosin which was filtered out and dissolved in alcohol. The resulting solution showed no chromatin staining properties upon dilution with water. Unna's polychrome methylene blue, which is rich in *roth aus methylene blau*, gave no precipitate with eosin until acetic acid was added and the precipitate thus obtained, dried and dissolved in alcohol, had no chromatin-staining properties. After repeated experimentation, Reuter concluded that Nocht's *roth aus methylene blau* cannot cause the chromatin stain in malarial parasites, that it is an indicator of the presence of the essential dye and should be regarded as an end product of the decomposition of methylene blue by alkali, whereas the nuclear stain is an intermediate product in this reaction. For the latter substance he suggests the name *A(alkali) methylene blau*. To make his staining solution Reuter precipitated Nocht's solution with eosin, washed the precipitate several times, and dried it in the dessicator. Two-tenths of a gram was dissolved in 100 c.c. ethyl alcohol and 2 c.c. anilin added. Thus prepared, the solution was permanent. To stain, he added 30 drops of this to 20 c.c. of water, immersed the preparation 20 to 30 minutes, sometimes three to four hours. Any precipitate on the preparation could be removed with absolute alcohol. Reuter regarded his dye as bearing no relation to Michaelis' methylene azure.

Leishman¹⁴ prepared and used his staining solution as follows:

Solution A.—To 1 per cent solution of medicinally pure methylene blue in distilled water add 0.5 per cent sodium carbonate and heat at 65° for 12 hours, then allow to stand 10 days at room temperature.

Solution B.—Eosin extra B. A. Grüber one to one thousand aqueous solution.

Mix solutions A and B in equal amounts and allow to stand six to twelve hours, stirring at intervals; filter and wash the precipitate thoroughly, collect, dry, and powder. Fifteen-hundredths gram is dissolved in 100 c.c. pure methyl alcohol to form the staining solution. It keeps perfectly (five months). The solution is a clear dark blue color and shows greenish iridescence by reflected light. To stain add three to four drops to an unfixed film, held in Cornet's forceps. After half a minute add six to eight drops of distilled water and mix by rotation. Allow to stain five minutes; then wash in distilled water and allow the water to remain on the specimen one minute. Examine in water, dry in air, and mount in balsam. The entire procedure requires seven to eight minutes. The after-treatment with water is an important part of the differentiation.

Wright¹⁵ prepared the polychrome solution by adding 1 per cent of methylene blue to a $\frac{1}{2}$ per cent sodium bicarbonate solution and steaming for one hour. When cold he precipitated this by the addition of a 1:1,000 eosin solution, collected the precipitate on a filter, and dried without washing. When thoroughly dry a saturated solution in pure methyl alcohol was made, filtered and diluted with 25 per cent of methyl alcohol. The staining is carried out as in Leishman's method.

Giemsa¹⁶ undertook the further investigation of the claim made by Michaelis that azure is the nuclear dye of the Romanowsky stain. Methylene violet (*Badische Anilin- und Soda-Fabrik*) alone showed no staining power in aqueous solution. He made a crystalline eosin compound with this methylene violet, dissolved it in alcohol, and attempted to stain preparations by diluting this solution with water. A rich precipitate resulted, but no staining. He next tested methylene azure. The crude dye (containing some methylene violet) gave no results worth mentioning, but with Höchst methylene azure pure, excellent stains were obtained. Yet this azure still contained traces of methylene violet. He therefore prepared chemically pure azure hydrochloride by a rather complicated process and obtained it as needle-shaped crystals from alcohol. A solution of this mixed with eosin gave most excellent Romanowsky stains, with the added advantage that the same mixture could be used repeatedly, provided only that an excess of azure was present. The preparations lacked only the blue color of the protoplasm, which was here more of a gray tone. Addition of an equal part of methylene blue to the azure did not detract from the chromatin staining, but produced the typical blue color in the protoplasmic portions of the preparation. Addition of methylene violet, however, prolonged the staining and caused a dirty precipitation on the film. Giemsa thus agrees with Michaelis that methylene azure is the essential and only red nuclear dye of the altered methylene blue, but he believes that the eosin also takes part in the chromatin staining.

The color reaction of dilute solutions with sulphuric acid, as given by Michaelis, Giemsa¹⁷ found untrustworthy for the determination of the relative quantities of methylene azure and methylene violet in a mixture. A mixture might contain 40 per cent methylene violet and still not be distinguished from pure azure by this test. A better test, in his experience, has been to add a few drops of fixed alkali to a dilute solution of the substance in question and shake it thoroughly with ether. If azure alone was present the ether became bright scarlet red. If methylene violet was also present the ether became more or less violet; in the absence of azure a pure violet color. The color of the ether should be compared with that of ethereal solutions of known chemically pure azure and violet. If undecomposed methylene blue was also present the water below the ether remained blue, if absent this was colorless.

Giemsa does not describe his method of making methylene azure, but the substance may be purchased from Grüber as "Azur I (pur.);" for five marks a gram. The mixture of it with methylene blue, equal parts, can be had for one-half this price, as "Azur II." It is used as follows. An 0.8 per cent solution Azur II and a 0.05 per cent eosin (Höchst extra water soluble) are made up. Ten c.c. of the latter are mixed with 1 c.c. of the azure blue solution and the fixed blood preparation immersed for 15 to 30 minutes, washed in water, and examined, dried, and mounted in acid-free balsam.

After many attempts to make a good staining solution by treating methylene blue with alkalis, Giemsa has concluded that this is practically impossible, for, whatever the conditions, methylene violet was always produced and the amount of methylene azure was relatively small, especially when heat was employed. Nocht's *roth aus methylene blau* he found to be a mixture of methylene-violet and methylene-azure bases. No way has been found to avoid the production of methylene violet in these solutions or to get rid of it after its formation. He made a solution of the azure-eosin compound in pure methyl alcohol, but only mediocre results were obtained with it while fresh, and upon standing it decomposed and failed to stain chromatin after a few weeks. He therefore condemned Reuter's stain which he considered to be a mixture of the eosin compounds of methylene blue, methylene violet, and methylene azure, the last alone being the active principle. In a later report Giemsa¹⁸ has simplified his method somewhat and now recommends the following preparation:

Azur II-eosin	3.0 g.
Azur II	0.8

finely ground and sifted through a fine sieve into 250 grams glycerin at a temperature of 60° C., dissolve; then add 250 grams methyl alcohol at a temperature of 60° C. Shake and allow to stand 24 hours at room temperature, filter. To stain, one drop of this solution is mixed with 1 c.c. of water and the fixed preparation immersed in it. The preparations are best fixed by pure methyl alcohol, three to five minutes. The addition of a few drops of very dilute alkali carbonate to the staining mixture intensifies the chromatin stain.

Unna²¹ undertook to ascertain the real constituents of his polychrome methylene blue, inasmuch as Michaelis had made the statement that it consisted essentially of methylene azure. He obtained samples of methylene azure and several of its salts from Giemsa and tested it upon tissue sections. He found that its staining power was not equal to that of his polychrome methylene blue. Only when methylene violet was added to the azure solution did he get good preparations, and he therefore still regards methylene violet as a most essential constituent of polychrome methylene blue. He obtained the best results in staining tissue sections with the following mixture:

Azure carbonate (Giemsa)	0.25 g.
Potassium carbonate	0.25
Methylene violet (Bernthsen)	1.00
Glycerin and distilled water, equal parts, to	100.00 c.c.

Marino²² has recently modified methylene azure so that it may be used in methyl alcohol solution. The formula follows:

Methylene azure	0.50 g.
Methylene blue	0.50
Water	100.00 c.c.

Add 0.5 per cent sodium*carbonate and place for 24 to 48 hours at a temperature of 37° C. or better at a higher temperature. Then precipitate with eosin, determining by titration the exact amount of this to be added. Filter and obtain a powder; 0.04 gram of this is dissolved in 20 c.c. methyl alcohol. This is used the same as Leishman's solution except that 0.005 per cent eosin solution is used in place of the distilled water to dilute the alcoholic solution on the cover-glass.

In a recent paper Giemsa²⁰ opposes Marino's modification for the same reason that he condemned Reuter's stain. In this paper he announces that he will soon publish in *Les folia hematologica* a complete compendium of the literature bearing upon the Romanowsky stain.

May²³ has observed that blood preparations stained in ordinary methylene blue and eosin may be changed into particularly good Romanowsky preparations by after-staining with a solution of Giemsa's Azur I or with a ripened alkaline methylene-blue solution. For some details the latter solutions gave better results than the pure azure and he thought it possible that this might be due to some other decomposition product of methylene blue.

From the brief review of these papers it is apparent that the nuclear dye of Romanowsky's stain depends upon some of the products of the treatment of methylene blue with dilute alkali, the necessary change taking place more quickly at higher temperatures. These products are fairly well known, thanks to the pioneer work of Bernthsen and his pupils, but his method of preparing the two apparently most important ones is so difficult and expensive that few have had opportunity to test them. Although one worker has discovered a method of making methylene azure much better than that of Bernthsen, he has not chosen to share this important knowledge, so that investigators are still dependent upon the old difficult method. The other product, methylene violet, though easily made by Bernthsen's method, is difficult to purify, and those who have tested it are not agreed as to its properties.

The disputed question of the essential constituents and the action of the Romanowsky stain can be entered upon only after these constituents have been recognized and prepared in a pure condition. There is no agreement in the literature upon this question. Michaelis has identified the essential nuclear dye of the Romanowsky stain as methylene azure, while Reuter has convinced himself that another substance "A(alkali)methylene blau," different from azure, is the important substance. Unna appears to stand alone in claiming that methylene violet acts as a stain, and he has not shown any relation between it and the Romanowsky stain.

Pure methyl alcohol, as a solvent for the dyes, has proven very valuable in the hands of several observers, yet Giemsa, with chemically pure azure and its derivatives at his command, has failed to prepare a useful solution in pure methyl alcohol, and condemns all such solutions.

To attempt to reconcile, confirm, or refute the various conflicting experimental results reported, and to perfect the practical staining procedure has been the double purpose with which our experiments were undertaken. Although these ends have not been accomplished in the desired degree, yet some of the results obtained appear of such importance that their confirmation by others is desirable, and, if they stand this test, they should mark an advance in our knowledge of the Romanowsky stain.

EXPERIMENTAL.

The first experiment was an attempt to isolate and identify Nocht's *roth aus methylene blau*, as this certainly seemed to bear some relation to the Romanowsky stain. Extraction with ether by separating funnel proved unsatisfactory as the yield was too small, so a mechanical extractor, somewhat like the Soxlet apparatus, was constructed from glass (Fig. 1) for this purpose. To 300 c.c. of 1 per cent methylene blue (med. pur.), 1.5 grams of sodium carbonate crystals were added and the solution heated on a boiling-water bath one-half hour. It was then cooled and placed in the bulb of the extractor and extracted with ether. The ether, in passing up through the Nocht solution, took on a deep cherry-red color, and, as the extraction proceeded, a solid ring collected on the wall of the receiving flask at the surface of the boiling ether. The extraction was continued for 10 hours, at the end of which time the ether was coming away with a pale reddish-yellow tint and a considerable ring had already collected on the flask below.

The apparatus was disconnected, the ether poured off, and the extract dried in an air current and scraped out. It weighed 0.15 gram and was dark purple, almost black, in color. It was almost entirely insoluble in water; under the microscope the undissolved flakes appeared as dense black masses. Where the light penetrated they were seen to be red-purple in color. In warm, dilute hydrochloric acid the substance dissolved with a blue color to reprecipitate on cool-

ing as yellowish-black needles (hydrochloride). The substance dissolved in concentrated sulphuric acid with a violet-blue color. The last two reactions identified it as nearly pure methylene violet (Bernthsen). From this it would appear that the decomposition of methylene blue by sodium carbonate and heat produces considerable methylene violet and only small amounts of methylene azure and that Nocht's *roth aus methylene blau* is chiefly methylene violet base. A solution of this ethereal extract and eosin in methyl alcohol failed to stain nuclei appreciably, the red color being very faint, thus confirming Reuter, who failed to get chromatin to stain with the ethereal extract. But the addition of a small amount of methylene blue (med. pur.) to the solution made an excellent staining reagent, the nuclei taking the deep purple-red color characteristic of the Romanowsky stain.

The extraction by ether of a fresh, 1 per cent methylene-blue solution containing $\frac{1}{2}$ per cent sodium carbonate removed only a slight amount of coloring substance, the ether showing only a faint pink tint. When instead of sodium carbonate, $\frac{1}{2}$ per cent sodium hydroxide was added and the extraction performed in the absence of air (extractor), the methylene blue was almost entirely decomposed in 24 hours, retaining only a pale blue tint. A black precipitate was thrown down in it. In the receiving flask below a considerable amount of dark brown substance had been deposited, the ethereal extract. After drying in a current of air this was found to be very soluble in water; it failed to form crystals with hydrochloric acid. By the test given on p. 427, methylene violet, methylene azure, and methylene blue were easily recognized in it. The last was apparently



FIG. 1.

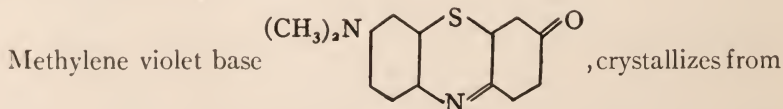
extracted as a leuko-base and oxidized during the subsequent drying.

Methylene violet was next prepared by Bernthsen's method (p. 412), purified by repeated crystallization from alcohol, and finally obtained as green, shining, needle crystals almost insoluble in water, soluble in concentrated sulphuric acid with a violet-blue color, and forming with dilute hydrochloric acid long needles soluble in warm, but insoluble in the cold dilute, acid.

A saturated alcoholic solution of this substance failed entirely to stain nuclei of blood cells when applied alone or diluted with water or watery eosin solution. In the presence of a little methylene blue it stained nuclei the characteristic purple-red color. After considerable experimentation this method of making methylene violet was simplified somewhat, and I now make it as follows:

Methylene blue (med. pur.)	4 g.
Sodium carbonate (crystals)	4
Distilled water	2,000 c.c.

Dissolve the sodium carbonate separately and add to the methylene-blue solution. Boil for five to ten hours, filter and wash the precipitate with water. It consists of long, needle crystals of methylene violet, often pure enough to form crystals with hydrochloric acid. This product I designate as crude methylene violet. It may be used for staining (see p. 427). There is considerable impurity, however, chiefly methylene blue and methylene azure, and to get pure violet it is necessary to recrystallize from alcohol repeatedly. In this way beautiful green, shining crystals are obtained. In making methylene violet from methylene blue, a dilute solution is essential, as otherwise the precipitate begins to form while there is still a large amount of undecomposed methylene-blue base present, and the product is correspondingly rich in this substance, from which it is quite difficult to separate the methylene violet.



alcohol in opaque needles with a bright green luster, permanent in the air. It is insoluble in cold water, tints warm water a faint blue, dissolves slowly in ether with scarlet-red (cherry-red) color, not violet as

stated by Giemsa. It dissolves in methyl and ethyl alcohol with deep violet-blue color and rich red fluorescence. The formation of clean-cut needle crystals from alcohol is the best test for purity. Although insoluble in water, it is quite soluble in methylene-blue solution from which it may be extracted by ether even in the presence of an excess of carbonic acid. It is a weak base and appears to form no carbonate. It forms no compound with eosin in alkaline or neutral solutions, but in faintly acid solutions it forms a red-purple compound insoluble in water. This is at once decomposed by dilute sodium carbonate solution with formation of the free methylene-violet base. In concentrated sulphuric acid methylene violet dissolves with a violet-blue color, in strong hydrochloric acid likewise with a blue color. In fairly dilute (20 per cent) hydrochloric acid it dissolves when warmed and crystallizes out in long needles as the hydrochloride upon cooling. These crystals are soluble in water and in concentrated hydrochloric acid.

Methylene-violet hydrochloride was prepared by dissolving the pure base in warm, 1 per cent hydrochloric acid, filtering, and precipitating the dye by the addition of sodium chloride. The precipitate was washed on the filter with a saturated solution of sodium chloride several times and finally with pure water. Apparently the substance dissociated readily in the absence of free hydrochloric acid, for a strong aqueous solution gave a deep red color to ether without the addition of alkali. The crystalline form was also destroyed to a certain extent by washing with the salt solution. It was dried at a temperature of 55° C. *in vacuo* and finally obtained as a dark brown powder, soluble in water with a red-violet color, but immediately precipitated as amorphous red flakes by the addition of sodium carbonate.

An important property of methylene violet is that of combining with methylene blue. If 0.5 gram methylene violet and 0.5 gram methylene blue be dissolved in 100 c.c. of water by means of a few drops of hydrochloric acid and this solution then made slightly alkaline with sodium carbonate, it at once fills with crystals and the solution becomes quite pale violet in color. These crystals appear the same as those formed by boiling methylene blue with sodium carbonate in dilute solution, and consist of a combination or intimate mixture

of methylene violet and methylene blue. They are slightly soluble in water with a violet color. When a larger proportion of methylene blue is used as, for example,

Methylene-violet hydrochloride	0.2 g.
Methylene blue	0.8
Distilled water	100.0 c.c.

the addition of sodium carbonate no longer causes precipitation and the solution remains deep blue in color.

An attempt was also made to obtain methylene azure of Bernthsen. The filtrate from the preparation of methylene violet by Bernthsen's method was rendered slightly acid and then stannous chloride and alkali added until the color was completely reduced and the reaction strongly alkaline. It was then placed in the bulb of the mechanical extractor and extracted with ether. This fulfilled quite well the conditions given by Bernthsen, as the solution was completely protected from air. The ether came away colorless after the apparatus had been in operation a short time, but when this colorless ether was exposed to the air, it became red and a blue precipitate was deposited on the glass. Evidently the ether contained a substance which, when oxidized, became insoluble in ether. This led me to believe that the extract contained leukomethylene violet and leukomethylene blue, the latter precipitating as methylene blue upon exposure to the air while the violet remained in solution as the red color. The extraction was continued, and finally the ether poured out of the receiver and the deposited extract was dried by a current of air. During this process it probably took up oxygen and carbon dioxide. It was then found to be very easily soluble in water; dilute alkalis (fixed) caused immediate change from blue to purple color in the solution with precipitation of the color base; it failed to form crystals with hydrochloric acid and thus corresponded to some extent to the descriptions of methylene azure. Yet it was certainly mixed with methylene violet. Another lot of the solution was reduced and extracted in the same way, the receiving flask being changed after the first hour. It was found that the first portion was fairly rich in the red coloring substance, while that in the second flask was very poor in this color, its watery solution changing color somewhat upon the addition of alkali, but remaining much more blue than in the case of the first. This showed that the

ethereal extract varied in its qualitative composition, which would of course, be impossible if it contained a single substance. It evidently consisted of methylene azure, methylene violet, and methylene blue, extracted by ether as their leuko-bases. The separation of methylene azure from methylene violet gave Bernthsen and Simon considerable trouble, and Simon considered it quite possible that their purest azure still contained methylene violet. Giemsa also found that the yield of azure was very small by Bernthsen's methods of preparation. Pure methylene azure has been made by this investigator, but inasmuch as he has not published his method, it is impossible to follow his work. From his papers one would judge that his method of making it is a fairly complicated and expensive one.

Accepting the composition of methylene azure given by Bernthsen, I attempted to make it by treating methylene blue with hydrogen peroxide in acid and alkaline solutions, and with hydrogen peroxide and ferric chloride in acid solution, without result. By the use of chromic acid in acid solution, however, indications of azure formation were obtained at the first trial, as was also the case when potassium permanganate and acid were used. The following method was finally decided upon, and I shall endeavor to describe it so that anyone with a little chemical training can make pure methylene azure. Thirty-five c.c. of hydrochloric acid (Sp. G. 1.20) is diluted to a liter with distilled water and 10 grams of Ehrlich's rectified methylene blue added and dissolved by heat. To the boiling solution 35 c.c. of a hot, 10 per cent aqueous solution of potassium bichromate is slowly added with constant stirring. A precipitate of methylene blue chromate forms in the solution, but as the boiling continues this redissolves with a deep blue color. The boiling is then continued until all the methylene blue has been converted into azure, as determined by the test here given. After ten minutes' boiling a few drops of the solution are removed to a test tube and diluted slightly with cold distilled water. A dilute solution of sodium hydroxide is then added drop by drop, shaking the test tube until the color has changed from blue to red. About 5 c.c. of ether is then added and the tube vigorously shaken a few moments and then allowed to settle, when the ether will have taken up the azure base and, if methylene blue is present, a blue tint will remain in the water below. The test should

be performed quickly, else a small amount of methylene blue may not be detected. This test is repeated at short intervals until methylene blue is no longer present. Then the hot solution is filtered and nearly saturated with sodium chloride and allowed to stand for about two hours. The azure hydrochloride precipitates as irregular bunches of small plate crystals, which are filtered out and recrystallized from alcohol to purify. To do this the precipitate is placed in a flask and a half liter of alcohol added, thoroughly mixed, heated to boiling on the water-bath, and filtered. The filtrate is then boiled, distilling off the alcohol until the dye begins to separate out of the boiling solution, whereupon it is poured out into a clean beaker and set aside to crystallize. These crystals are then filtered out, redissolved in fresh alcohol, and the operation repeated. It is finally obtained as long, hairlike crystals of a green color which become brown upon drying *in vacuo* at a temperature of 55°C . The yield is about three grams. When dissolved in water, treated with a drop of dilute alkali and extracted with ether, pure azure leaves no blue color in the water below. If the supernatant ether, containing the azure base is poured off into a tube containing sodium bicarbonate solution and shaken with this, the ether is decolorized and the water becomes blue. The base has been changed to the carbonate and as such is insoluble in ether.

I have not undertaken an ultimate analysis of this substance, but its origin from methylene blue by action of oxidizing agents, its correspondence in properties with the description given by Bernthsen, and its identity in chemical and staining reactions with "Azur I pur.," Giemsa, purchased from Gröbler, led me to believe that it is methylene azure. The amount of bichromate used in making it is just slightly in excess of the theoretical amount required to furnish two atoms of oxygen to each molecule of methylene blue and this agrees with Bernthsen's analysis according to which the composition of the iodide is $\text{C}_{16}\text{H}_8\text{N}_3\text{SiO}_2$.

One of the most characteristic properties of methylene azure salts is the immediate complete change of color, upon the addition of free alkali, from blue to red, due to the setting free of methylene azure base, which is only very slightly soluble in water. This is a much stronger base than methylene violet, rapidly taking up carbonic acid

from the air to form a carbonate which is quite soluble in water with a blue color. Upon this property is based the method of detecting and separating methylene violet and methylene azure when present in the same solution (see below).

Methylene azure, like methylene blue, decomposes when heated with dilute alkali carbonates, with the formation of methylene violet and some other, undetermined substances. Methylene azure 0.5 gram, sodium carbonate 0.5 gram, distilled water 200 c.c., were heated over the direct flame and boiled 15 minutes. Even before the boiling point was reached a precipitate had begun to separate, and at the end of 15 minutes' boiling, the solution was quite pale. It was cooled somewhat and filtered, the filtrate giving a strong test for methylene violet. The precipitate consisted chiefly of thin crystals and dissolved partly in warm, dilute hydrochloric acid. This solution upon cooling formed long, needle crystals, methylene-violet hydrochloride.

Methylene azure-eosin may be formed by mixing strong alcoholic solutions of azure hydrochloride and sodium eosin. It crystallizes as fine purple needles, insoluble in water, slightly soluble in alcohol. It is also formed by mixing water solutions of these two dyes. Recrystallization from alcohol failed; apparently boiling alcohol decomposes the substance.

Methylene azure and methylene violet are both present in polychrome methylene blue, and as free bases both are soluble in chloroform with a deep red color. In ether, methylene-violet base has a deep pinkish-red color, raspberry red; azure base dissolves with a yellowish-red or brownish-red color. The latter is a strong base, combining readily with the carbon dioxide of the air to form a carbonate. Methylene violet-base, on the other hand, is set free from its salts by sodium bicarbonate or by calcium carbonate, and may then be extracted by ether. To test a given solution of ripened alkaline methylene blue for the presence of these substances, the method of Michaelis is insufficient, and the test given by Giemsa is inaccurate. The color of methylene violet in ether is not violet, but scarlet-red. The following test is an improvement. Dilute the unknown solution until it is nearly transparent in a test tube, add a few drops of 1 per cent hydrochloric acid and then an excess of solid sodium bicarbonate. Carbon dioxide should be evolved. If no bubbles appear, a few more

drops of the acid should be added. The addition of the acid serves to neutralize any previous free alkali in the solution, either free dye base, alkali hydroxide, or carbonate, and to saturate the solution with carbonic acid. The solution is now shaken with ether and a red color indicates methylene violet. By repeatedly extracting with fresh quantities of ether this is entirely removed. If now an alkali hydroxide is added to the solution and the ether extraction repeated, it now removes methylene azure, which has been held back in the water as a carbonate, and assumes an eosin-red color (yellowish in dilute solution). If methylene blue is absent the water is now completely decolorized, if present the water remains blue-tinted for a time. The test is not sharp here, for even dilute alkalis decompose the methylene blue so that a small quantity of this dye in the original solution may escape detection by this method. In doubtful cases it is well to use a fairly strong solution of the unknown and rapidly to extract both the red bases together (by adding alkali hydroxide and shaking with ether) and then note the color of the water. When properly performed one can easily detect all three dyes in Unna's polychrome methylene blue.

Methylene violet* may be used as a stain in either alcoholic or aqueous solutions. Alone, however, it does not stain as has been abundantly shown by previous workers. A saturated solution in methyl alcohol used as such, by diluting with distilled water, or with eosin solution shows no staining power. If, however, an equal amount of methylene blue be present the characteristic colors of the Romanowsky stain are produced. The following solution has proven quite satisfactory for general blood staining.

Methylene violet (pure crystals)	0.08 g
Methylene blue (med. pure)	0.16
Eosin (water soluble yellowish) Grüber	0.20

Powder and mix thoroughly and dissolve in 100 c.c. of warm pure methyl alcohol. Cool to room temperature, filter and dilute with 10 c.c. methyl alcohol. This solution is used as a fixing and staining

* The dye list of Schultz and Julius, Berlin, 1897, mentions methylviolett, $C_{20}H_{19}N_4Cl$, dimethylsaffranin chloride. Several brands of this substance are on the market under this trade name, methylviolett, and they have been examined. It is a substance entirely different from Bernthsen's methylene violet and should not be confused with it. Recently two samples of the latter have been obtained. One bearing the label "Methylviolett v. Bernthsen, Dr. G. Grüber & Co., Leipzig," is evidently an altered methylene blue, strongly alkaline, but contains only a trace of methylene violet. The other sample, labelled "Bernthsen's Insol Methylene Violet, E. Leitz, New York and Chicago," yields crystals with dilute hydrochloric acid and seems to be about as pure as the crude methylene violet which I have prepared. In methyl alcohol solution made up according to the formula given, it is a satisfactory stain.

solution according to Leishman's method. I have experienced very little trouble from its running off the coverglass during the staining. For this purpose it has been found convenient to bend the blades of the Novy forceps so as to eliminate the tendency of the stain to run up between them. It can be done easily by anyone; the figure explains itself. The bend is so slight that the forceps may still be used



FIG. 2.

to pick up coverglasses from a flat surface. This staining solution seems to keep indefinitely in a well-stoppered bottle and, if by evaporation it becomes too concentrated, a little methyl alcohol may be added from time to time. I have used one solution at intervals for three months and it is still as good as when first prepared.

Pure methylene violet is not necessary to make a good methyl alcohol stain, for I have obtained good results by using the crude violet (see p. 427) which anyone can easily make. It should be quite dry before use. The following formula has given good results:

Methylene violet (crude)	0.08 g.
Methylene blue (med. pur.)	0.08
Eosin (water soluble yellowish)	0.20

These are dissolved in 100 c.c. methyl alcohol, filtered, and diluted as in the previous solution. The violet used here contains some methylene blue, traces of azure, and other undetermined substances.

These solutions, it seems to me, furnish the Romanowsky stain in the most convenient, surest, and best form for the use of physicians and clinical laboratories.

In staining films from syphilitic lesions, a very dilute solution of sodium carbonate (1:15,000) should be substituted for the distilled water ordinarily used to dilute the stain in Leishman's method.

For aqueous solution the methylene violet hydrochloride is employed, being mixed with an excess of undecomposed methylene blue and then rendered alkaline and allowed to stand a few days before using. For staining malarial parasites and trypanosomes the following solution has proven of value:

Methylene violet hydrochloride	0.1 g.
Methylene blue (med. pur.)	0.9
Glycerin	50.0 c.c.
Distilled water	40.0

Dissolve and add 10 c.c. of a 5 per cent sodium carbonate solution. After standing a few days to allow the alkali to set free the dye bases the solution is used in the same way as that of Nocht, by adding a few drops to 2 to 10 c.c. of a very dilute (1:10,000) solution of eosin. The exact proportions need to be determined by experiment, and therefore the method is not recommended for clinical work. For staining spirochetes a solution stronger in methylene violet has, so far, seemed better.

Methylene violet	0.2 g.
Methylene blue	0.8
Glycerin	50.0 c.c.
Distilled water	40.0

Dissolve and add 10 c.c. of 5 per cent sodium carbonate.

The use of these solutions presents the same difficulties met with in using Nocht's solution, but the staining is better. I hesitate to recommend them as I think the best combination has not yet been attained. Some surprisingly good preparations have been obtained with them, however. The preparations must be fixed before staining, either in absolute alcohol and ether, or in pure methyl alcohol (three to five minutes) as suggested by Giemsa. Preparations of spirochetes stain more intensely if placed for a few minutes in a 0.05 per cent solution of sodium carbonate and then rinsed just before staining. One may gain some idea of the intensity of the stain from the fact that often *Sp. Obermeieri* may be easily seen with the low power (Leitz Obj. No. 3, Oc. No. 1) after it is stained by this method. A tendency to formation of precipitate upon the preparation is a drawback, yet in some of the most intensely stained preparations this is absent.

The mode of action of methylene violet is somewhat problematical, but it seems most probable that the real dye penetrates and stains as a combination of methylene blue and methylene violet, "A(lkali) methylene blau" of Reuter, and that the methylene-blue constituent is then gradually removed by the action of eosin, leaving finally the red violet-base in the nucleus. In accord with this is the fact that methylene violet forms no combination with eosin in alkaline solution, and staining takes place only in alkaline solution.

These stains have been tested upon human malarial blood, blood of leukemia, pernicious anemia; blood from rats, mice, and guinea-pigs infected with *Trypanosoma Brucei*; from rats with *Tr. Lewisi*, from rats infected with *Sp. Obermeieri*; upon smears from primary and secondary luetic lesions.

The use of methylene azure in alcoholic solution has been found impracticable by Giemsa. I have tried the following solution:

Methylene azure hydrochloride	0.1 g.
Methylene blue (med. pur.)	0.1
Eosin, water soluble, yellowish	0.1
Methyl alcohol	100.0 c.c.

Much of the dye remained undissolved on the filter. The resulting solution gave fairly good stains of blood elements, malarial parasites and trypanosomes, not so good in the case of spirochetes. The solution gradually deteriorated, until at the end of two months no nuclear stain could be obtained with it in the ordinary way, only the red blood cells being stained with the eosin. But the substitution of very dilute sodium carbonate solution (1:10,000) for distilled water as the diluting fluid produced fairly good nuclear staining again. The gradual decomposition of the azure-eosin in methyl alcohol would seem, therefore, to give rise to acid, quite probably formic acid from oxidation of the solvent, and the deterioration of the solution is due to the fact that this acid prevents the dye from acting and not to the decomposition of all the azure-eosin, as has been supposed by Giemsa.

Marino's modification (p. 418) contains some alkali in the solution as made up, and so does not become useless so quickly. It will probably be found to deteriorate with age, however, as not all the azure has been decomposed by his preliminary heating.

With methylene azure in aqueous solution it is possible to obtain excellent stains of hematozoa. The various methods have been quite thoroughly tested by Giemsa, and I have gone into this part of the work only far enough to convince myself that his claims for azure are well founded. The preparations are remarkably free from precipitation. The color of the nuclear stain produced is, however, not exactly the same as that obtained by the method of Nocht or the numerous modifications of it. Certain details are brought out better by azure than by the older procedures or by methylene violet-blue solutions;

but certain other details are not, for example, the blood platelets and spirochetes. The following watery solution has proved very useful:

Methylene azure hydrochloride, pure	0.5 g.
Methylene blue (med. pur.)	0.5
Sodium carbonate	0.25
Glycerin	50.0 c.c.
Distilled water	50.0

The constituents dissolve readily. The solution is used by adding a few drops to 10 c.c. of very dilute (1:5,000) eosin solution and immersing or floating the fixed preparation on the mixture for 10 to 15 minutes. The resulting stain is excellent. Giemsa believes that the nuclear color is a combination of azure and eosin, while Michaelis regards it as due to the azure alone. I am inclined to regard Michaelis' view as the more correct, for a solution of methylene azure alone, rendered slightly alkaline with sodium carbonate, stains nuclei of leucocytes the red-purple color. Differentiation of detail is, however, not so good as when eosin is also used.

In general, methylene azure does not seem to stain chromatin so intensely nor to outline it so sharply as do the violet-blue solutions. Further experimentation is necessary to determine the best conditions for staining with the latter, especially in aqueous solutions. Mixtures of methylene violet with methylene blue and azure have been tested, but so far seem to offer no advantages.

The composition of ripened-alkaline methylene blue must be regarded as a variable one, both methylene violet and azure being among the constituents. If ripened at a high temperature very little of the latter is present, and methylene violet is the prominent staining factor. This is the case with most of the Romanowsky staining solutions recommended in the literature. By ripening at room temperature both the red dyes are produced.

A mere trace of methylene violet in a methylene blue solution is sufficient to cause the red chromatin stain when the solution is combined with eosin in the proper proportion. For instance a solution of

Methylene violet hydrochloride	0.005 g.
Methylene blue (med. pur.)	0.995
Sodium carbonate	0.25
Distilled water	100.00 c.c.

if combined with the proper amount of dilute eosin gives the red chromatin stain. The staining process requires about an hour.

In conclusion I take great pleasure in acknowledging my indebtedness to Professor G. Carl Huber, Director of the Histological Laboratory, for the incentive to undertake this work and for constant counsel and encouragement during its progress. I am also under obligations to Professor F. G. Novy for important suggestions and the loan of valuable apparatus and materials, and for the blood parasites used in testing the stain, and to Dr. H. A. Freund for films of pathological human blood.

LITERATURE.

1. BERNTHSEN. *Liebig's Ann. d. Chemie*, 1885, 230, p. 137.
2. SIMON. *Inaug. Diss.*, Zurich, 1885.
3. KEHRMANN UND SCHAPOSHNIKOFF. *Berichte der deutschen chem. Gesellsch.*, 1897, 30, p. 1571.
4. ROMANOWSKY. St. Petersburg, 1891. Cited by Nocht (7).
5. UNNA. *Ztschr. f. wiss. Mikros.*, 1891, 8, p. 447.
6. ZIEMANN. *Ueber Malaria und andere Blutparasiten*, Jena, 1898.
7. NOCHT. *Centralbl. f. Bakt.*, 1898, 24, p. 839.
8. NOCHT. *Ibid.*, 1899, 25, p. 17.
9. NOCHT. *Ibid.*, 1899, 25, p. 764.
10. JENNER. *Lancet*, 1899, 1, p. 370.
11. MICHAELIS. *Centralbl. f. Bakt.*, 1901, 29, p. 763.
12. REUTER. *Münch. med. Wchnschr.*, 1901, p. 48, 1261.
13. REÜTER. *Centralbl. f. Bakt.*, 1901, 30, p. 248.
14. LEISHMAN. *Brit. med. Jour.*, Sept. 21, 1901, 2, p. 757.
15. WRIGHT. *Jour. Med. Res.*, 1902, 7, p. 138.
16. GIEMSA. *Centralbl. f. Bakt.*, 1902, 31, p. 429.
17. GIEMSA. *Ibid.*, 1902, 32, p. 307.
18. GIEMSA. *Ibid.*, 1904, 37, p. 308.
19. GIEMSA. *Deutsche med. Wchnschr.*, 1905, p. 31, 1026.
20. GIEMSA. *Ann. de l'Inst. Pasteur*, 1905, 19, p. 346.
21. UNNA. *Monatsheft f. prakt. Dermatol.*, 1904, 38, p. 119.
22. MARINO. *Ann. de l'Inst. Pasteur*, 1904, 18, p. 761.
23. MAY. *Münch. med. Wchnschr.*, 1906, 53, p. 358.

ARE OPSONINS DISTINCT FROM OTHER ANTIBODIES?*

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WRIGHT and Douglas first demonstrated that normal serum contains certain substances, called by them opsonins, which render various bacteria susceptible to phagocytosis by polynuclear leucocytes. Wright and his followers, Neufeld and Rimpau, as well as others have shown that the substances arising in response to immunization promote phagocytosis by a similar opsonic action upon the bodies that are taken up by the phagocytes.¹

Whether this opsonic action is the property of antibodies with which we are already somewhat familiar, such as amboceptors and agglutinins, or whether opsonins are distinct and independent units in the serum, is an interesting and important question.

Before the exact interaction of serum and cells in phagocytosis had been made clear, Metchnikoff and his followers attributed the promotion of phagocytosis by immune serum to the so-called fixators, which in general are regarded as identical with Ehrlich's amboceptors. Quite recently Dean,² from the results of the study of the effects of heat upon the opsonic power of normal and immune serum, has expressed the view that amboceptors may exercise the functions of opsonins, which consequently cannot be regarded as independent substances. Neufeld and Töpfer,³ however, as well as Barratt,⁴ hold that the opsonic substances for erythrocytes—hemopsonins—that arise in animals on immunization with alien blood are distinct from the hemolytic amboceptors, because a serum might be lytic for certain corpuscles without being opsonic, and vice versa.

In the course of our work a number of facts have been brought out that seem to be of importance in regard to the question of the entity of the opsonins.

* Received for publication March 25, 1906.

¹ I have discussed phagocytosis and opsonification in various phases in the Middleton-Goldsmith Lecture of the New York Pathological Society, February 23, 1906. (*Jour. Am. Med. Assoc.*, 1906.)

² *Proc. Roy. Soc.*, B, 1905, 76, p. 506, and *Centralbl. f. Bakt.*, Abt. I, 1905, Ref. 37, p. 349.

³ *Centralbl. f. Bakt.*, Abt. I, 1905, 38, Orig., p. 456.

⁴ *Proc. Roy. Soc.*, B, 1905, 76, p. 524.

In the first place I have shown¹ that the opsonic effect of dog serum upon anthrax bacilli is practically destroyed, at most residual traces only being left, on heating the serum to 60° to 62° C., whereas the amboceptor for anthrax bacilli in dog serum is not appreciably affected by heating to 65° C. for 30 minutes. This appears to indicate that the opsonin and the amboceptor in dog serum are distinct bodies with different degrees of resistance to heat, but of course it cannot be denied that the amboceptor might act as opsonin and that the opsonic function might be the first to be lost on heating. Hence it is necessary to examine into the conditions presented by other sera.

The serum of white rats is normally anthracidal, due to a thermostable substance that is neutralized by neutralization of the serum with oxalic acid. The same serum contains an opsonin for anthrax bacilli which, however, is not neutralized by oxalic acid.²

A somewhat analogous condition obtains in the serum of rabbits and goats immunized against the so-called virulent pseudodiphtheria bacilli, studied by Miss Hamilton in conjunction with Miss Horton.³ Here the bactericidal substance is also thermostable, and as yet in no way resolvable into amboceptor and complement. These sera also contain specific opsonins which differ in important points from the bactericidal substances.

While it cannot be asserted that the amboceptor nature of the opsonins in these instances is absolutely excluded, there is good reason to regard them as distinct substances.

Normal human serum, as well as other sera, contains opsonins for streptococci and pneumococci, but not any lytic amboceptors for these organisms, which grow freely in the sera. Besredka and Dopter⁴ applied the Bordet-Gengou method of fixation of the complement in order to learn whether the serum of scarlet-fever patients contains a special fixator (amboceptor) for any special streptococci, but in no one of seven cases could they demonstrate fixators for streptococci cultivated either from the patient furnishing the serum or from other sources. The Bordet-Gengou method is regarded by the French workers as a reliable method for demonstrating specific

¹ *Jour. Infect. Dis.*, 1906, 1, p. 102.

³ *Jour. Infect. Dis.*, 1906, 1, p. 128.

² HORTON, *Jour. Infect. Dis.*, 1906, 1, p. 110.

⁴ *Ann. de l'Inst. Pasteur*, 1904, 18, p. 373.

amboceptors in sera that are not bacteriolytic.¹ Now the serum of scarlet-fever patients contains as a rule considerable opsonin for streptococci as well as pneumococci, and consequently we may conclude that in this case there is no evidence to show that the opsonin is not a distinct substance.

Immunization with streptococci and with pneumococci has been shown by Neufeld and Rimpau and others to give rise to opsonins in the sera of the immune animals, but not, so far as we know, to the formation of specific amboceptors demonstrable by lysis of the organisms in question through the interaction of complement and amboceptor. It is true that Besredka² maintains that he has demonstrated specific fixators in some antistreptococcus sera by means of the Bordet-Gengou phenomenon. Without entering on a discussion of the reliability of the method, concerning which I feel that there is reason for some doubt, it may be pointed out that, while Besredka noted that heated streptococci did not fix the complement as well as the unheated, yet heated streptococci are opsonized by human serum as readily as the unheated.

Greig Smith³ observed that agglutinating serum causes phagocytosis of typhoid bacilli which are not taken up by washed leucocytes in NaCl solution. I have found that normal serum is opsonic for typhoid bacilli, but not necessarily agglutinating. Naturally opsonized bacilli may be taken up in large numbers when in clumps, but mere clumping, as by vesuvin, safranin, etc., does not lead to phagocytosis (Greig Smith). In the case of other bacteria also, e. g., streptococci, pneumococci, anthrax bacilli, opsonification may be induced by various sera that have no agglutinative effect upon the particular bacterium in question.

The opsonic substances possess greater power to resist heat than do most of the complements with which we are familiar. Human

¹ Bordet and Gengou (*Ann. de l'Inst. Pasteur*, 1901, 15, p. 288) devised an ingenious method by which it is claimed that the presence of special fixators (amboceptors) may be demonstrated in sera that are not bacteriolytic. The serum to be tested is first heated and then mixed with a small quantity of fresh normal serum—complement—and bacterial emulsion. After standing for six hours at the room temperature red corpuscles treated with heated hemolytic serum are added. If there is no hemolysis, it is held to mean that the complement in the fresh serum which is suitable for lysis of properly prepared corpuscles has been absorbed by the bacteria by virtue of their having taken up special amboceptors present in the serum tested. This is the phenomenon of "fixation of the complement."

² *Ann. de l'Inst. Pasteur*, 1904, 18, p. 363.

³ Abstr. in *Bull. de l'Inst. Pasteur*, 1906, 4, p. 44, from *Proc. of Linnean Society of New South Wales*, 1905.

serum, normal as well as that obtained from convalescents from typhoid fever, retains its opsonic action on typhoid bacilli after complete destruction of the complement by heat. For these and other reasons it is not likely that opsonic action is exercised by complements as that word is understood in general at present.

By way of summary the following facts, then, speak in favor of the conclusions that bacterio-opsonins are distinct from other antibodies:

1. Heat may destroy the opsonic power, or opsonin, of serum, leaving the lytic amboceptors intact.
2. Serum, normal as well as immune, may contain opsonin for a given organism but not, at least so far as is yet known, the proper amboceptor for that organism.
3. A serum may contain opsonin for a bacterium but no agglutinin, and the opsonin may persist after the bacteriolytic complement has been destroyed by heat.

Strong evidence in favor of the view that opsonins constitute a distinct class of substances is furnished by the study of phagocytosis of red corpuscles.

As indicated already Neufeld and Töpfer regarded the opsonin for red corpuscles—hemotropic substance as they would call them—produced on immunization with goat blood as a distinct body, because hemolytic and agglutinating sera are not necessarily opsonic.

The sera of various animals contain amboceptors in fairly pronounced concentrations for a variety of heterologous erythrocytes, but it is only exceptionally that a trace of erythrocytic opsonin can be demonstrated in normal sera. Now it may be urged that normal amboceptors really carry opsonic powers, but that this has not been made evident because proper leucocytes have not been used as phagocytes. This objection is not without some force, because there is reason to believe that opsonic serum may subject erythrocytes as well as bacteria to phagocytosis by one kind of leucocyte but not by other kinds.

What may be learned from study of the serum of animals immunized with alien blood?

Barratt¹ has observed that doves immunized with hen blood

¹ *Loc. cit.*

yield a serum which is strongly opsonic with respect to hen corpuscles, but not at all lytic. He also notes that various immune hemopsonic sera are devoid of agglutinative effect on the corresponding corpuscles. Barratt consequently believes in the entity of opsonin.

In the serum of rabbits immunized with goat corpuscles there develops the new power to lake goat corpuscles (amboceptors) and to render the corpuscles of various animals, but specifically those of the goat, susceptible to phagocytosis by dog and other leucocytes (opsonin). The immune rabbit serum, however, does not possess increased hemolytic or agglutinative power with respect to all these corpuscles. The immune amboceptor for goat corpuscles is readily activated by normal rabbit serum; consequently there is little reason to believe that the presence of amboceptors for other corpuscles cannot be demonstrated because of the lack of proper complement, and the most reasonable conclusion is that immune opsonin is distinct from the amboceptors; the more so in view of the fact that on several occasions I have been able to demonstrate the presence of opsonin in the serum in which heating to 70°C .—the serum being undiluted—for one hour seemed to have destroyed completely the hemolytic amboceptor for goat corpuscles.

Furthermore, by means of absorption experiments, I have been able to separate the amboceptor from the opsonin for goat corpuscles in the serum of rabbits immunized with goat blood. First I determined that 0.0125 c.c. of the particular serum at my disposal constitutes what may be called an adequate opsonic dose for 0.2 c.c. of a 5 per cent suspension of washed goat corpuscles, using 0.2 c.c. of a suspension of washed leucocytes (dog or guinea-pig) to furnish the phagocytes. An easily demonstrable phagocytosis of goat corpuscles now takes place within an hour after putting the mixtures in the incubator.* This amount of heated immune serum (0.0125 c.c.) also causes complete laking of 0.2 c.c. of a 5 per cent suspension of goat corpuscles when activated with 0.05 c.c. of normal rabbit serum. The amboceptor concerned in this laking may be removed completely by means of washed sheep corpuscles, the opsonin for

* It must be remembered that in these experiments it is not possible to determine degrees of phagocytosis with the same accuracy as when we use bacteria, the number of which taken up may be counted and averaged.

the goat corpuscles being left behind. The complete experiment with the necessary controls is illustrated in Table 1.

TABLE 1.

SEPARATION OF AMBOCEPTOR AND OPSONIN FOR GOAT CORPUSCLES IN SERUM OF RABBIT IMMUNE TO GOAT BLOOD.

Immune serum 0.1 + 5 per cent Washed Sheep Corpuscles 1.6 c.c. Centrifugate after One Hour at 37° C.

LYSIS.

Supernatant fluid 0.2 (0.0125 im. ser.) + 5% washed goat corpuscles 0.2 + normal rabbit serum 0.05	=No lysis
Immune serum 0.0125 in NaCl sol. 0.2 + 5% washed goat corpuscles 0.2 + normal rabbit serum 0.05	=Complete lysis
NaCl sol. only 0.2 + 5% washed goat corpuscles 0.2 + normal rabbit serum 0.05	=No “
NaCl sol. only 0.2 + 5% sheep corpuscles treated as above with immune serum 0.2 + normal rabbit serum 0.05	=Complete “
NaCl sol. only 0.2 + 5% normal sheep corpuscles 0.2 + normal rabbit serum 0.05	=Trace “

PHAGOCYTOSIS.

Supernatant fluid 0.2 (0.0125 im. ser.) + 5% washed goat corpuscles 0.2 + susp. washed dog leucocytes	=Phagocytosis
NaCl sol. only 0.2 + 5% sheep corpuscles treated as above with immune serum 0.2 + susp. washed dog leucocytes	= “
NaCl sol. only 0.2 + 5% normal goat corpuscles 0.2 + suspension washed dog leucocytes	=No “
NaCl sol. only 0.2 + 5% normal sheep corpuscles 0.2 + suspension washed dog leucocytes	= “ “

TABLE 2.

ABSORPTION OF OPSONIN IN SERUM OF RABBITS IMMUNE TO GOAT BLOOD BY (1) SHEEP CORPUSCLES AND (2) GOAT CORPUSCLES.

1.

Immune Serum 0.05 + 5 per cent Suspension of Washed Sheep Corpuscles 2.4 c.c. Centrifugate after One Hour at 37° C.

Supernatant fluid 0.6 (0.0125 of serum) + 5% susp. goat corpuscles 0.2 + suspension dog leucocytes 0.2	=Good phagocytosis
Supernatant fluid 0.6 (0.0125 of serum) + 5% susp. sheep corpuscles 0.2 + suspension dog leucocytes 0.2	=No “
Immune serum 0.0125 in NaCl sol. 0.6 + 5% susp. sheep corpuscles 0.2 + suspension dog leucocytes 0.2	=Good “

2.

Immune Serum 0.05 + 5 per cent Suspension of Washed Goat Corpuscles 2.4 c.c. Centrifugate after One Hour at 37° C.

Supernatant fluid 0.6 (0.0125 of serum) + 5% susp. sheep corpuscles 0.2 + suspension dog leucocytes 0.2	=No phagocytosis
Supernatant fluid 0.6 (0.0125 of serum) + 5% susp. goat corpuscles 0.2 + suspension dog leucocytes 0.2	= “ “
Immune serum 0.0125 in NaCl sol. 0.6 + 5% susp. goat corpuscles 0.2 + suspension dog leucocytes 0.2	=Marked “

Table 1 shows that sheep corpuscles absorb opsonin as well as amboceptors in the immune serum, and the question consequently arises whether there are two opsonins in the serum, one for goat

corpuscles and one for sheep corpuscles, or whether the sheep corpuscles remove only a fraction of a single opsonin. Further absorption experiments show that while goat corpuscles in excess remove all the opsonins in the serum for both kinds of corpuscles, sheep corpuscles in excess appear to remove only opsonins peculiar to themselves, as shown in Table 2. This result indicates that immune opsonic serum may contain specific and group opsonins analogous to the specific and group agglutinins in the sera of animals immunized with certain bacteria. The further discussion of this phase of the subject must be postponed to some future time.

The facts, then, which indicate that the erythrocytic opsonins also are distinct substances are, in brief, the following:

1. Normal serum may contain hemolytic amboceptors but not hemopsonins.
2. Immune serum may contain opsonic substances but not amboceptors or agglutinins for the corpuscles in question.
3. By absorption methods the specific amboceptors in an immune serum may be separated from the specific opsonins.
4. The opsonic power of serum persists after the complement has been destroyed by heat.

Finally, in view of the principal facts brought forward, namely, that normal serum may possess lytic power but not opsonic, and vice versa; that immunization in some cases may give rise to opsonic substances, but not to lytic amboceptors or to agglutinins; and that the specific amboceptor has been separated from the specific opsonic substance in an immune serum, it may be concluded that the opsonic function of normal and immune serum is due to a distinct body for which opsonin is an apposite and convenient name.

SOME OBSERVATIONS ON PHAGOCYTOSIS OF DIPH- THERIA BACILLI*

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CONTRARY to the statement of Wright and Douglas¹ that *B. diphtheriae* is not readily taken up by the leucocytes in normal serum, the strains under my observation were found very susceptible to phagocytosis. When a rather thick suspension of bacilli is incubated for about one hour with an equal quantity of normal defibrinated human or dog blood, as many as 50, or even more, bacilli may be taken up by a single leucocyte. Two strains of *B. diphtheriae*, both virulent to guinea-pigs, were tested. Leishman's quantitative method of estimating the degree of phagocytosis was employed. As a rule, the various mixtures were kept in the incubator for one hour. When more than about 10 bacilli are taken up by a single leucocyte it is difficult to make an accurate count, especially when the bacilli have become fragmented or only the granules are distinctly stained. Hence rather thin suspensions were usually found preferable. From 20 to 50 leucocytes were counted to get the average number of bacilli taken up.

1. When the blood serum is removed by washing in normal salt solution, phagocytosis is greatly diminished or almost absent, depending chiefly on the thoroughness of the washing. Four or even five washings are required. Even after such thorough washings a few leucocytes often appear to have taken up a considerable number of bacilli, while the majority are quite inactive. This is oftenest found when very concentrated suspensions are used. It often appears as if a *clump* of bacilli—the presence of a few clumps is difficult to avoid in preparing suspensions of *B. diphtheriae*—had settled upon the leucocyte, rather than that such a number had actually been taken up by the leucocyte.

In the following set of experiments, in each of which a different blood and a different suspension of bacilli were used, a portion of

* Received for publication March 28, 1906.

¹ *Proc. Roy. Soc.*, London, 1903, 72, p. 357.

the blood was washed four or five times, and enough normal salt solution was added to make up the same bulk as before washing; 0.3 c.c. of normal blood and washed blood were then placed in separate tubes and incubated with equal amounts of the same bacterial suspension.

HUMAN BLOOD.		DOG BLOOD.	
Unwashed	Washed	Unwashed	Washed
33.00	5.70	12.45	4.00
17.30	5.40	6.50	0.02
5.25	0.76	3.10	0.00
11.30	0.80		

2. When normal serum is added to washed leucocytes, phagocytic activity is largely restored. This does not occur if the serum has been previously heated to 58 to 60° C. for 15 to 30 minutes.

	PHAGOCYTOSIS			
	Unwashed	Washed	Washed plus Normal Serum	Washed plus Heated Serum
Dog blood.....	6.00	0.20	2.45	0.60
Human blood.....	11.30	0.80	8.50	0.10

3. When the bacilli have been suspended in normal serum for 30 minutes at 37° C., the serum being subsequently removed by washing they undergo phagocytosis by washed leucocytes. The bacilli are not thus sensitized by serum which has been heated at 58° to 60° C. for 30 minutes. It was found that when the bacilli were treated with normal serum, there was an average phagocytosis of 9.5 by washed dog leucocytes, while the same quantity of the same bacterial suspension, when treated by the same quantity of heated serum gave a phagocytosis of only 0.6. Similarly with washed human leucocytes, two different suspensions treated with normal serum gave respectively a phagocytosis of 7.0 and 23.4, while, under identical conditions, treatment with heated serum gave 0.1 and 4.5 per leucocyte.

4. E. L. Walker¹ reported that when diphtheria bacilli are heated at 70° to 100° C., there is a decrease in phagocytosis, and he ascribed this to a destruction of the toxin by heat. An attempt was made to repeat the experiment, and, if the result had been verified, it was

¹ 'The Relative Influence of the Blood Fluids and the Bacterial Toxins on Phagocytosis,' *Jour. Med. Res.*, 1905, 14, p. 173.

planned to restore phagocytosis by means of diphtheria anti-toxin. However, no marked decrease in phagocytosis was observed after heating the bacilli. Heating the bacilli at 70° to 75° C. for one-half hour, and then incubating with normal human blood, gave the following results, smears being examined at the end of every hour for four hours:

	1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.
Unheated bacilli.....	19.0	31.0	50.0?	75.0?
Heated "	13.5	22.0	20.0	35.0

It will be seen that after one hour's incubation there is only a slight difference in the phagocytosis of the heated and unheated bacilli. The greater differences after longer periods of incubation are probably due to an increase in the number of the unheated bacilli, and a consequent real or apparent greater phagocytosis. Heated bacilli also often stain poorly, and this may result in a lower count; possibly some are entirely destroyed by prolonged heating.

When rather concentrated suspensions were used, so many bacilli were taken up, even after heating to the boiling-point, that an accurate count was found impossible, as in the following experiment: Similar quantities of a uniform suspension were heated 15 minutes each, at temperatures varying from 60° to 100° C., and then incubated one hour with normal blood; there was no apparent increase or decrease in phagocytosis as a result of the heating; even the bacilli that had been boiled were taken up in such numbers that an accurate count was impossible. This experiment was repeated several times with *B. diphtheriae* and also with *St. aureus*.

5. Some tests were made of the opsonic power of the blood serum of diphtheria patients. Two series of tubes were prepared, each tube containing 0.2 c.c. of a suspension of washed human blood and 0.2 c.c. of a suspension of diphtheria bacilli; then to one series were added falling quantities of normal human serum, to the other series similar quantities of serum from the diphtheria patient, enough normal salt solution being added to make up a total of 0.6 c.c. in each tube. Serum from a patient seven days ill, with temperature slightly above the normal and a few bacilli still in the throat, gave the following result:

	Normal Serum	Serum from Diphtheria Patient
0.2 c.c. serum.....	44.0	34.0
0.1 "	37.0	29.0
0.05 "	37.5	28.3
0.025 "	28.5	19.5
0.006 "	22.0	24.2

There appears to have been some decrease in opsonin at this time. After another week there was a further decrease; the patient exhibited some symptoms of pulmonary tuberculosis (a positive diagnosis being made soon after), and no further tests were made in this case.

The serum of another patient was tested after two weeks' illness. The temperature was normal; bacilli were still found on the tonsils. In this case a rather marked increase in opsonin was present.

	Normal Serum	Serum from Diphtheria Patient
0.2 c.c. serum.....	4.20	6.05
0.1 "	3.60	4.02
0.05 "	2.30	3.30
0.025 "	2.90	4.10
0.006 "	1.00	2.20

It would be of interest to determine conclusively whether or not there is an increase in opsonin during convalescence from such a disease as diphtheria, where the infective organisms multiply only locally, while the toxins invade the general system. This, if demonstrated, would tend to show that some substance is split off by the bacteria which enters the circulation and stimulates the formation of opsonin. It would be desirable to follow several cases with this end in view through the whole course of the disease.

It might also be of interest to follow the opsonic index of the horse or other animal during a process of immunization with diphtheria toxin. Two such observations have been made on the horse, one during the earlier course of the immunization, the other after some degree of immunization had been established. The first observation, conducted in the same way as the experiments on the human patients, showed a decrease in opsonin, the index being approximately 0.5. In the second the index was found to be about 1.0.

CONCLUSIONS.

1. *B. diphtheriae* is very susceptible to phagocytosis.
2. Phagocytosis is not materially affected by heating the bacilli, as claimed by Walker, and it has not been shown that the diphtheria toxin favors phagocytosis.
3. There is probably an increased formation of opsonin during convalescence from diphtheria, but this point requires further investigation.

LATENT PNEUMOCOCCEMIA.*

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ALTHOUGH much work has been done on pneumococcal infections and pneumococemia our knowledge of the mechanism of these processes is still in its infancy. It is only within recent years that it has been established by Fraenkel,¹ Prochaska,² and others, but especially Rosenow,³ that in every case of pneumococcal pneumonia we have a pneumococemia. Heretofore pneumococemia was believed to occur practically only in fatal cases, and accordingly the entrance of the pneumococcus into the general circulation was viewed with grave apprehension. With the establishment of this pneumococemia the question naturally arises, is this general invasion primary and the pulmonary process secondary, simply a localization at the *locus minoris resistentiae*; or is the reverse true and the breaking through into the blood stream but a natural consequence of the local involvement. Experimentally Washburn,⁴ Fraenkel and Schultz⁵ were able to produce pulmonary conditions identical with those found in man by means of intravenous and intraperitoneal injections, and inclined to the former view. Rosenow was able to verify their results and, moreover, succeeded in demonstrating the pneumococcus in the blood in five cases before any local physical signs were present.

Intimately associated with this problem is the question, What is the fate of the pneumococci in the blood, especially with relation to crisis? Clinically the pneumococcal joint localizations, usually post-critical, as pointed out by Herrick,⁶ clearly show that the pneumococcus can persist in the blood after crisis. Its virulence in this case is indicated by the fact that it does produce pathological processes. The

* Received for publication March, 29, 1906.

† This work has been carried out by means of the Fellowship Fund of the Alumni Association of Rush Medical College.

¹ *Deutsche med. Wchnschr.*, 1901, 27, p. 298.

² *Deut. Arch. f. klin. Med.*, 1901, 70, p. 559; *Centralbl. f. inn. Med.*, 1900, 21, p. 1145.

³ *Jour. Infect. Dis.*, 1904, 1, p. 280.

⁴ *Lancet*, 1902, 163, p. 301.

⁵ *N. Arch. d. Sci. biol. d. St. Petersburg*, 1901 8, p. 1.

⁶ *Am. Jour. Med. Sc.*, 1902, 124, p. 1234.

same may be said of pneumococcal endocarditis and many other postcritical metapneumonic processes.

We have but few isolated facts at hand bearing on this intricate problem. Baudel¹ in 1899 obtained the pneumococcus from the blood as late as 25 days after crisis. Prochaska demonstrated the pneumococcus in the blood four times after crisis; on the day after crisis in one uncomplicated case, and in one complicated by serous pleuritis; two days after crisis in a case complicated by muscle abscess; three days after crisis in a case of delayed resolution. Rosenow in his series of 145 cases, examined eight cases from 4 to 36 hours after crisis, finding the pneumococcus four times, i. e., in 50 per cent; more recently three unpublished cases within 24 hours after crisis. Recently Tizzoni and Panichi² found the organism in five cases, 30, 31, 31, and 56 days, respectively, after crisis and over 15 months after convalescence. They also demonstrated pneumococci in "hyper-vaccinated" rabbits 11 months after injection.

During the past winter I have made careful observations in regard to this question on 21 cases of lobar pneumonia chosen at random. Blood cultures were made in broth and milk. In all the cases one or more cultures were made before crisis and usually several cultures were made on successive days after crisis.

The pneumococcus was recovered after crisis in six of the 21 cases of lobar pneumonia. It was not obtained before crisis in five of the remaining 15 cases, and therefore these should be set aside as not comparable. Thus the pneumococcus was isolated after crisis in six out of 16 cases, or in 37 per cent of the cases showing the organism before crisis.

BRIEF STATEMENT OF CASES.

1. Age 15 months. Typical left lobar pneumonia. Pneumococci in the blood. Crisis five days after admission to the hospital. Culture from the blood eight days after the temperature had become normal gave pneumococci.

2. Male, age 40, alcoholic. Typical left upper lobe pneumonia. Crisis occurred on same day, but three days later the temperature became variable (96.8°-100°) with persistent leucocytosis. Fifteen days later had a chill with a rise of temperature, returning to normal the next day. Patient had a chill on three successive days, the

¹ *Rif. Med.*, 1899, 11, p. 170.

² *R. Accad. d. Sc. di Bologna*, 1905, jan. 15; *Rendiconti Accad. dei Lincei*, XIV, 2, série, 16 juillet, 1905, pp. 107-14; 6 août, 1906 (Abst. *Bull. de l'Inst. Pasteur*, 1905 3 p. 420); *Centralt. f. Bakt.*, 1905 36, pp. 25-47.

temperature returning to and remaining normal 20 days after the original crisis. A blood culture secured during the second chill (17 days after the crisis) yielded abundant growth of pneumococci. Delayed resolution present.

3. Colored, age 34, cook. Typical left lower lobe pneumonia. Temperature remained high for 11 days after admission, then became irregular for six days when it became normal. Positive cultures were obtained four days after admission and again two days after his temperature had become normal. Delayed resolution present.

4. Male, age 24, colored. Typical left lower lobe pneumonia. Crisis occurred eight days after admission, but two days later temperature again rose and remained irregular (97.4° – 103°), until the 15th day after crisis. Signs of delayed resolution present. Cultures before crisis and 16 days after crisis gave positive results.

5. Male, age 31, laborer. Typical left lower lobe pneumonia with extension to left upper lobe seven days after admission. Crisis 10 days after admission. Blood cultures before crisis and eight hours after crisis were positive.

6. Male, age 39, machinist, ward man in the hospital. Taken suddenly with right lower lobe pneumonia. Blood cultures three hours after chill sterile, but cultures taken 24 hours after chill and again eight hours after crisis, which occurred on the third day, gave pneumococci.

We see that the pneumococcus was found six times after what appeared to be a typical crisis; three times eight hours after, and in three cases complicated by delayed resolution 7, 16, and 17 days after crises, respectively, and one to two days after temperature finally remained normal. No attempt was made to ascertain how long after crisis the organisms could be obtained.

CHARACTERISTICS OF ORGANISMS OBTAINED FROM THE BLOOD AFTER CRISIS.

Tizzoni and Panichi claim that the organisms they isolated after crisis were non-virulent and atypical morphologically, resuming, however, typical appearances after a short period of cultivation. These organisms, they assume, had been changed, maintaining an inoffensive existence in the blood. I have not been able to reach the same conclusions from the study of the organisms obtained by me after crisis.

Morphology.—The organisms obtained in all cases were typical lance-shaped, encapsulated pneumococci, grouped usually in pairs. All fermented inulin and also formed the characteristic green zone about the colonies on blood agar.

Virulence.—Four strains were tested for virulence by intraperitoneal injection of 24-hour broth cultures of rabbits with the following results:

TABLE 1.

	Case	Dose	Time of Death
Rabbits, weight 2,000 to 2,500 grams.....	1	3-5 c.c.	24-36 hours
" " 1,700 to 1,900 "	2	4-5	24 "
" " 1,700 "	5	4	36 "
" " 1,300 "	6	5	48 "

We see that all the organisms tested show considerable virulence for rabbits, giving results similar to those obtained with organisms isolated from the same patients before crisis.

Relation of the organisms to phagocytosis.—Metchnikoff in his early studies of phagocytosis made the important observation that virulent streptococci were not susceptible to phagocytosis. More recently the same results have been obtained with virulent pneumococci. Moreover, it has been found by Rosenow that this resistance to phagocytosis as well as the virulence is readily lost by artificial cultivation under favorable conditions, but can be again restored, although with difficulty, by repeated passages through animals. Because of this intimate association of virulence and resistance to phagocytosis, it would seem reasonable to expect that the organisms considered in the foregoing would be relatively insusceptible to phagocytosis. Hence the experiments whose results are given in Table 2 were made.

TABLE 2.
ABSENCE OF PHAGOCYTOSIS OF ORGANISM FROM CASE 1.

PNEUMOCOCCAL SUSPENSION+ BLOOD OR SERUM+WASHED BLOOD CORPUSCLES	PHAGOCYTOSIS (20 LEUCOCYTES COUNTED AT END OF ONE HOUR)	
	Pneumococcus Case 1 (Isolated after Crisis)	Avirulent Pneumococci
Human blood (defibrinated).....	o	25+
Human serum 0.25+guinea-pig leucocytes 0.25.....	o	25+
Guinea-pig exudate (whole) 0.5.....	o	25+
Dog blood (defibrinated) 0.5	o	23
Dog serum 0.25+washed human corpuscles 0.25	o	25+

The above table shows that the strain isolated from Case 1 after crisis is not susceptible to phagocytosis by the leucocytes in normal human blood, dog blood, or guinea-pig exudate, or by guinea-pig leucocytes in human serum or human leucocytes in dog serum. As normal sera were used in the above experiments, it might be objected

that even though these organisms were not sensitized by normal serum they readily become susceptible to phagocytosis when treated with the serum of the patient harboring the organisms. With this in view the following experiment was made, the result showing that the pneumococcal strains were not taken up by the phagocytes in normal blood nor in the blood from the patients from whom the organisms were isolated.

TABLE 3.

ABSENCE OF PHAGOCYTOSIS OF PNEUMOCOCCI IN BLOOD FROM PATIENTS FROM WHOM THEY WERE OBTAINED.

Blood+Pneumococcal Suspension	Phagocytosis (20 Leucocytes Counted after 1 Hour)
Blood Case 2 after crisis + pneumococcus Case 2 isolated after crisis	0
Blood Case 2 after crisis + nonvirulent pneumococcus'	21
Blood Case 5 before crisis + pneumococcus Case 5 isolated before crisis	0
Blood Case 5 before crisis + nonvirulent pneumococcus	7
Blood Case 5 after crisis + pneumococcus Case 5 isolated before crisis	0
Blood Case 5 after crisis + nonvirulent pneumococcus	23.4
Blood Case 5 after crisis + pneumococcus Case 5 isolated after crisis	0
Blood Case 6 after crisis + pneumococcus Case 6 isolated after crisis	0
Blood Case 6 after crisis + nonvirulent pneumococcus	11

SUMMARY AND CONCLUSIONS.

The above results show that the pneumococcus does exist in the blood of a large per cent of the pneumonia patients after crisis. This is in accord with the findings of other observers. How long they do remain is as yet undetermined, perhaps a few hours or a few days in cases running a normal course to several weeks in cases complicated by delayed resolution, endocarditis, or some other metapneumonic process. Whether they can exist in the blood of uncomplicated cases for weeks or even years as Panichi states is, to say the least, highly questionable. Pneumococci isolated after crisis are virulent for rabbits to the same degree as are the precritical organisms derived from the same patients. Virulence is also indicated by the fact that a pneumococcic strain is insusceptible to phagocytosis by normal human and dog blood and guinea-pig exudate; furthermore, by the facts that other strains show no evidence of phagocytosis when treated with normal blood nor with blood drawn from the homologous patient after crisis, when we would expect the opsonic index to be at its highest.

We are therefore forced to conclude that crisis cannot be identical with a sudden disappearance of all organisms from the blood of a

pneumonia patient. It would be more reasonable to assume that crisis marked the point where the increase is more than counter-balanced by the destruction of the organism, presumably by phagocytes. (Further reference will be made to this point in another paper.) Neither do the experimental results nor the clinical evidence appear to me to permit us to assume that the pneumococci at the time of crisis acquire some mysterious properties which render them nonvirulent and harmless and yet insusceptible to phagocytosis.

In view of the fact that during the course of pneumonia a number of metabolic products are more readily formed (acids, ferments, and the like), locally as well as in the blood, the chemistry of which is wholly unknown, it would seem better not to connect the phenomenon of crisis too closely with the presence of the pneumococcus in the blood.

I herewith wish to express my thanks to the Rush Alumni Association for making the work possible; to Dr. Hektoen, under whose direction it was done; and to the house staff of the Cook County Hospital for the many courtesies shown.

THE ETIOLOGY AND DIAGNOSIS OF HYDROPHOBIA.*

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INTRODUCTION.

DURING the Spring of 1904 the "Negri bodies" were demonstrated by one of us in smears from the central nervous system of animals dead from hydrophobia. At that time, however, the technic was poor and the stains were unsatisfactory, so the use of the method in diagnosis was not begun. Many of the cases reported by Dr. Poor were studied by us in this way, the "bodies" being demonstrated in smears from three horses and from several dogs and guinea-pigs, while they were not found in normal dogs, guinea-pigs, or rabbits, or in guinea-pigs dead from tetanus or diphtheria toxin.

Last fall, in connection with the study of smears from vaccinia and variola stained by Giemsa's method, smears from hydrophobia cases were again tried and it was found that the "bodies" were brought out very clearly and characteristically by the Giemsa solution; and, as a consequence, the present work was planned.

Some of the most interesting material used by us has been obtained through the kindness of Dr. R. J. Wilson and of a number of veterinarians of New York City, to all of whom we wish to express our thanks.

We also wish to thank Dr. Poor and Dr. van Gieson for assistance in making some of the smears and sections from diagnosis animals, as well as for valuable suggestions.

Most of the sections have been prepared by Miss C. R. Gurley. The microphotographs were made in the Loomis Laboratory of Cornell University by Dr. M. Tracy, to whom we wish to express our

* Received for publication April 12, 1906.

indebtedness. All of the work has been done with the help and encouragement of Dr. Wm. H. Park, to whom thanks are due.

HISTORICAL REVIEW.

Investigations on hydrophobia have been carried on from three principal standpoints; first, the therapeutic; second, the diagnostic; and third, the etiological. Since the establishment of the Pasteur treatment, the importance of making a quick diagnosis has become so evident that the efforts of many workers have been directed toward this end, and only occasionally has the purely etiological standpoint been considered.

Pasteur and his immediate followers relied for their diagnosis entirely upon rabbit inoculations, and this meant a 15 to 20 days' wait before the patient knew whether or not the treatment he was receiving was necessary. In 1898 this time was shortened to about nine days in our laboratory by Wilson, who found that guinea-pigs came down with the disease much more quickly than rabbits. From time to time it has been thought that certain histological findings were diagnostic; for instance, the "rabid tubercles" of Babes, and the areas of "round and oval-celled accumulation in the cerebrospinal and sympathetic ganglia" of Van Gehuchten and Nelis, were said to be specific, but further study has shown that they are not absolutely specific for rabies. In many cases the whole picture of the grosser histological changes is sufficiently characteristic to warrant the diagnosis of rabies, but often it is not so. Bailey, in his studies on the ganglion cells in normal and hydrophobic rabbits gives a good bibliography of the histological findings up to 1901.

It was not until Negri, in 1903, described certain bodies seen by him in the large nerve cells in sections of the central nervous system that anything was found which seemed absolutely specific for hydrophobia. Negri claims that these bodies are not only specific for rabies, but that they probably are animal parasites, and the cause of the disease.

He describes them as usually round or oval bodies from 1μ to 23μ long, and containing vacuoles in some of which are granules of varying size and number; generally there is a central larger structure surrounded by smaller ones. By Mann's method of staining, the organism generally takes a brilliant eosin-red, with the exception of the granules, some of which stain a light blue, and the others a faint red. The central structure gives the appearance of being a nucleus. The bodies are sometimes in touch with the nucleus of the host cell, sometimes far from it, often in the cell branches where they are more elliptical in shape. There are irregular, pear-shaped, and three-cornered forms, all of which special shapes Negri thinks due to the position of the organism within the host cell. He speaks of multiplying forms but does not describe any definite division forms. He says he is able to identify the bodies in the hanging-drop fresh, and in a weak acetic acid solution; but does not recommend this method for general use in diagnosis, as it is difficult to differentiate the bodies under these conditions from the nerve-tissue elements. He finds his organism generally in greatest numbers in the large nerve cells of Ammon's horn, less frequently in those of the cerebral cortex, the cerebellum, the medulla, the spinal cord, and the cerebrospinal ganglia. The organisms vary greatly in numbers in the different cases. In some cases he could find only an occasional one, while in others they were innumerable.

He says very little about the bodies in animals dying of fixed virus, merely stating

that they have been found in rabbits that have died on the seventh day after inoculation with fixed virus, but that they are very tiny, infrequent, and found with difficulty. He finds the largest forms in dogs inoculated subdurally with street virus.

Negri's work was soon corroborated by many Italian observers. Volpino, Bertarelli, D'Amato, Daddi, Di Vestea, Guarnieri, and Martinotti, published almost immediately after Negri's first publication. They were soon followed by Celli and De Blasi, Pace, and Bosc. The "bodies" have been found by these authors, and later by others in all varieties of animals which are susceptible to hydrophobia, i. e., in dogs, cats, rabbits, rats, mice, guinea-pigs, birds, cattle, horses, and human beings.

In 1904 Luzzani published a report of 179 cases, and in all but nine of those which were proven by animal inoculation to be rabies, the "Negri bodies" were found.

In our own laboratory in the same year, Poor examined 19 cases of street rabies and many cases of experimental rabies, and in all except those inoculated with fixed virus the "bodies" were found. In fixed-virus animals he found an occasional homogeneous eosinophilic granule in the cerebellar cells, about which he expressed no opinion. Similar granules were also seen by various other observers, some of whom consider them possibly tiny forms of the organism; but nothing definite has been observed about them, and as indefinite granules have also been seen in other conditions, their significance is uncertain.

In 1904 Negri's work, so far as the presence of these "bodies" in hydrophobia is concerned, was further corroborated by the following workers: Dominici, Marzocchi, Bandini, Fasoli, and Schüder. There was no dissenting voice as to their presence, and as to their diagnostic value. Many controls were made by the different observers, especially by Volpino, Marzocchi, Dominici, and Poor. They examined the central nervous system of various animals that had died from poisoning with tetanus, strychnin, pneumococcus, staphylococcus, alcohol, formalin, tubercle bacillus, diphtheria toxin; and of human beings who had died from epilepsy, syphilis, alcohol poisoning, tuberculosis, and various nervous affections. Many normal animals were also examined, all with negative results so far as the "Negri bodies" were concerned.

During this time the "bodies" were tested for their resistance to various physical and chemical agents, such as heat, cold, drying, immersion in glycerin, etc., and they were found to retain their characteristic appearance and virulence after more or less manipulation. It does not necessarily follow, however, that the "bodies," even if they are living organisms, need to retain their characteristic appearance in order to be virulent. We know, for instance, that trypanosomes may seem to disappear from blood which continues to be infective. (Laveran and Mesnil.)

Remlinger showed that the medulla of rabbits inoculated subdurally with fixed virus may be virulent on the third day, but he did not work out the exact degree of virulence—that is, the approximate number of organisms in the material inoculated. The fact that it is virulent soon after inoculation, and that no "Negri bodies" have been found at this early period, he thinks is another indication that they are not organisms. He does not consider the possibility of there being tinier forms than those so far seen, but believes that the organism in its whole life-cycle is ultra-microscopic in size.

In regard to the significance of the "bodies," up to 1905 all of these authors, with two exceptions, agree with Negri in considering them probably Protozoa and the cause of hydrophobia. The two exceptions are Remlinger and Schüder.

These latter investigators consider the fact that the virus can be filtered through

a filter, practically impervious to ordinary bacteria, a proof that the "Negri bodies," which they say are too large to pass such a filter, are not the cause of hydrophobia. Bertarelli, however, showed that the residue after filtration was also virulent, and he and others expressed the opinion that besides forms too large to pass the filter there might be forms tiny enough to do so. We know that in a medium containing a growing protozoon we may find both large and small forms, the limits in size of the smallest forms not being known in some cases; the fact therefore, that the filtered portion and unfiltered solid residue both possess virulence is an added indication that we are dealing with Protozoa. MacNeal has shown with the trypanosomes that besides the large forms, there are forms tiny enough to pass a Berkefeld.

Practically nothing has been done with regard to the exact degree of virulence possessed by filtered and unfiltered portions of the emulsions of rabies virus. Late in 1905, Di Vestea showed that the filtered virus possesses characteristics different from unfiltered, thus indicating that the forms in each may be different in character. He thinks that the undiscovered extracellular forms may be tiny enough to pass the filter.

Quite recently Volpino elaborates more fully an hypothesis advanced by him in 1904, in regard to the filterable forms. He thinks that the real organism is very tiny, that probably only the inner bodies in the so-called "Negri body"—the tiny bodies which he had shown to be definite basophilic forms—are the parasites, and that the homogeneous-appearing substance in which they are imbedded and which makes up the rest of the "Negri body" as Negri describes it, is derived from the host cell, caused by the reaction of it to the parasite. He gives a number of drawings arranged in the form of a life-cycle to illustrate this idea.

Negri's latest article, appearing in June, 1905, states that the central body shows more characteristically as a nucleus in sections from rabid cattle which he had stained in a special way by hematoxylin, and that in the same animals there appear bodies presenting characteristics of cysts. These later studies confirm all of his previous work and emphasize the fact that some of the bodies contain a central complex characteristic mass of chromatin, sometimes appearing solidly stained, sometimes as a distinct network, and sometimes encircled by smaller solidly staining masses of chromatin. Each chromatin mass is surrounded by a clear, unstained ring.

The bodies which he interprets as cysts, he describes as similar in dimensions, shapes, and general staining characteristics to the other forms, but different in minute structure. By the staining method of Mann they seem to be filled with tiny, refractive, somewhat elongated granules. Some seem to be surrounded by a membrane which is occasionally notched as if about to break. The iron-hematoxylin stain brings out the structure of these bodies very clearly. They seem to be filled with numerous black-staining "spores" less than 1μ long and narrower, which appear as tiny filaments slightly curved with a small swelling near the center.

In 1905 still other workers corroborated Negri's work, among them Abba and Bormans, Way, Zaccaria, Maresch, Schiffmann, Galli-Valerio, and Bohne. Only one author failed to corroborate the work. Maas, in sections from a case of human rabies could find no "Negri bodies." Luzzani in this year published another collection of cases. Out of 457, 297 proved by the biological test to be hydrophobia, and in only nine of these were the "bodies" not found in sections. The bodies were not found in any other animal.

Maresch, by Bielschowsky's staining method, claims to have brought out the structure more distinctly.

Schiffmann, after studying the "Negri bodies" as they appear in street rabies and examining many controls, confirming fully the diagnostic value of the "bodies," studied the changes which they seemed to undergo in passage from animal to animal of the same species and of one species to another. He states that the greater the number of passages through a single species of animal, the smaller the "bodies," until in "fixed virus" in the rabbit no forms appear. He also says that he did not find any "bodies" in dogs inoculated with rabbit-fixed virus.

Bohne describes the shortest method so far published for examination of sections. The whole process lasts only three hours, and the author states that it is very satisfactory. The method is as follows: Small pieces of the nerve tissue are placed in 15 c.c. of pure acetone and kept at 37° C. for about 30 to 45 minutes. They are then put in 55° paraffin and left from 60 to 75 minutes, boxed, cut at 6 μ , dried at 60°, and stained with a modified Mann's method in 4 minutes. The "bodies" show a vacuolated and granular structure and some of the elliptical forms seem to be dividing. On the whole they take more of a magenta stain than the "bodies" do in sections prepared in the regular way. The author considers their parasitic nature still doubtful.

During 1905 a good review of recent studies on hydrophobia came out in the *Bulletin de l'Institut Pasteur*, and in 1906 Bertarelli published a good review in the "Referate" of the *Centralblatt für Bakteriologie*.

We may sum up the results obtained from the foregoing studies as follows:

1. In nearly 100 per cent of definite cases of street rabies characteristic "bodies" are found in the large nerve cells of sections from all or from a part of the central nervous system and the connected ganglia.

2. The general characteristics of most of these "bodies" are as follows: rounded or oval forms varying in size from 1 μ to 25 μ , with a homogeneous acidophilic ground substance containing a central body surrounded by granules; these inner bodies vary in structure and staining qualities, but are principally basophilic and may be in the form of reticular masses, rings, rods, or points; they are usually situated within vacuoles.

3. The "bodies" vary also in number, being very few in some cases, and numerous in others. According to one author they become fewer the greater the number of passages through a single species of animal, and are not found in fixed virus. Others have found occasional small forms in fixed virus, but not in large enough numbers to account for the infectivity of the nerve tissue.

4. No "bodies" have been found before the appearance of symptoms, although the central nervous system is infective before this time.

5. No "bodies" have been found in the peripheral nerves or in the salivary or other glands, although these organs have been shown to possess a certain amount of infectivity.

6. The most rapid satisfactory method of demonstrating the "bodies" for diagnosis is a complicated section process which takes at least three hours.

7. The filtered virus is infective, therefore some forms of the causative agent must be extremely tiny.

8. In no other disease have bodies similar in appearance to the "Negri bodies" been found.

9. When the "bodies" are found in sections, the diagnosis of hydrophobia is certain and the biological test need not be made; when they are not found, the case may have been one of hydrophobia and the biological test must be made.

10. The significance of the "bodies" is still in doubt for the following reasons: (a) They have not been found in all cases of hydrophobia, notably not in fixed virus, neither have they been found in all parts of nervous tissue proved to be virulent, especially before the beginning of symptoms; (b) forms small enough to pass the coarser Berkefeld filters have not been seen; (c) the structure has not been shown definitely to be analogous to that of known living organisms; (d) no definite series of forms indicating growth and multiplication have been demonstrated; (e) the staining qualities, contrary to those of known Protozoa, are more acidophilic than basophilic.

In January of 1906 one of the writers made a preliminary communication of part of the work reported in the following pages. Emphasis was placed upon the fact that the demonstration of the "Negri bodies" by the "smear method" which was recommended by the writer in 1904 (see discussion under Poor's first article) had, by better technic, proved to be wonderfully successful. By this method the structure of the "bodies" is brought out more definitely than by the section method, and the whole process is much simplified and may be completed within half an hour after removal of the nerve tissue from the animal.

The method of examining the central nervous system, especially

the brain, by smears has been used by several pathologists, among whom may be mentioned Ewing, who obtained interesting results by this method in his studies on the pathology of ganglion cells.*

ORIGINAL WORK.

The work may be divided into two parts:

I. The value of the "Negri bodies" in diagnosis and their rapid identification.

II. A study of the "bodies" with a view to determining their nature.

In all, 141 animals, including seven varieties, have been studied with these two points in view. The following table gives a classified list of these animals:

Street rabies cases	Dogs	25
	Cats	1
	Human beings . . .	3
Animals inoculated with street rabies	Dogs	7
	Rabbits	12
	Guinea-pigs	32
	Mice	5
Animals inoculated with fixed virus	Dog	1
	Rabbits	27
	Guinea-pigs	7
	Mice	1
Control animals	Dogs	12
	Rabbits	4
	Guinea-pigs	2
	Calf	1
	Human being	1

I.

In the first part of the work we have tried to determine: (1) Whether the "bodies" seen in the smears are similar to those seen in the sections, (2) the correspondence between the smear method, the section method, and the biological test, (3) the comparative value of each method in diagnosis, and (4) the specificity of the "bodies."

It was decided that these points might be brought out by using all three diagnostic tests in a series of street rabies animals and of a number of controls. Therefore with each animal chosen for this purpose

*Just after this paper went to press the article by Frothingham appeared. His work corroborates the results obtained by the smear method of diagnosing rabies. We have tried the impression method which he describes, as well as a number of other methods of making smears of the central nervous system and find the results obtained by them all good in some particulars, but the method we describe has so far given us uniformly better results in the diagnosis work.

the following routine was carried out: (1) the brain, medulla, and parts of the spinal cord and connected ganglia were removed; (2) small pieces from each part were fixed in Zenker's fluid; (3) smears were made from corresponding parts; and (4) animals were inoculated subdurally with an emulsion of corresponding parts, and from the animals that died either smears or sections or both were made.

The technic of the smear work is as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of oily substances.

2. A small bit of the gray substance of brain chosen for examination is cut out with a small sharp pair of scissors and placed about one inch from the end of the slide, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible.

3. A cover-slip placed over the piece of tissue is pressed upon it until it is spread out in a moderately thin layer, then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the smear and producing more well-spread nerve cells. If any thick places are left at the edge of the smear, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass. If the first smear does not seem to be well spread out others should be made until a satisfactory one is obtained.

4. For diagnosis work such a smear should be made from at least three different parts of gray matter of the central nervous system: first, from the cortex in the region of the fissure of Rolando or in the region corresponding to it (in the dog the convolution around the crucial sulcus), second, from Ammon's horn, third, from the cerebellum. In many of the animals reported here smears were made from the gray matter of the cerebral cortex, around the fissures of Rolando and Sylvius, from the olfactory bulb, Ammon's horn, cerebellum, medulla in the region of the roots of the cranial nerves, spinal cord in the dorsal and lumbar regions, spinal and Gasserian ganglia, salivary glands, suprarenals pancreas, and some of the peripheral nerves. From the last four-named structures the smears were not very successful, so only a few were made.

5. The smears are dried in air,* and subjected to one or both of the two following staining methods:

- (a) Giemsa's solution. The smears are fixed in methyl alcohol (commercial is just as good as pure) for about 5 minutes. The staining solution recommended

* This method has proved so practical in our hands that an effort is being made to extend its usefulness.

The Board of Health of New York City is preparing a circular containing a description of the foregoing technic with more explicit directions in regard to the regions from which the smears are to be made with the added information that such smears, as well as the fresh material, may be sent to the nearest laboratory familiar with the appearance of the "Negri bodies" or to the Research Laboratory of the N. Y. Health Department. If the smears have been made successfully and the "Negri bodies" are found, the sender may receive word almost immediately and no sections or inoculations of the material need be made.

last by Giemsa* (1 drop of the stain to every c.c. of distilled water made alkaline by the previous addition of one drop of a 1 per cent solution of potassium carbonate to 10 c.c. of the water) is poured over the slide and allowed to stand for one-half to three hours. The longer time brings out the structure better, and in 24 hours well-made smears are not overstained. After the stain is poured off, the smear is washed in running tap water for one to three minutes, and dried with filter paper. If the smear is thick, the "bodies" may come out a little more clearly by dipping in 50 per cent methyl alcohol before washing in water, then the washing need not be as thorough. By this method of staining, the cytoplasm of the "bodies" stains blue and the central bodies and chromatoid granules stain a blue-red or azur. Generally the larger "bodies" are a darker blue than the smaller, the smallest of all may be very light (Plate 19, Figs. 3-56). The stain varies somewhat according to the thickness of the smear. Some have a robin's-egg blue tint but this is after a longer fixation in the methyl alcohol. In this case the red blood cells may have a greenish tint. (See Part II for full description of "bodies" stained by this method.) The cytoplasm of the nerve cells stains blue also, but with a successfully made smear the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azur, the nucleoli a dull blue, the red blood cells a pink-yellow, more pink if the decolorization is used. The "bodies" have an appearance of depth, due to their slightly refractive qualities.

For diagnostic purposes this method of staining may be shortened as follows: Methyl alcohol, 5 minutes, equal parts of the Giemsa solution and distilled water, 10 minutes. In this way "bodies" are generally brought out well enough for diagnosis, and sometimes the structure shows distinctly. It is always well, however, to make smears enough for the longer method of staining, in case the shorter one should prove unsatisfactory.

(b) The eosin-methylene blue method recommended by Mallory. The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap water they are placed successively in 95 per cent alcohol + iodine one-quarter hour, 95 per cent alcohol one-half hour, absolute alcohol one-half hour, eosin solution 20 minutes, rinsed in tap water, methylene-blue solution 15 minute, differentiated in 95 per cent alcohol lasting from one to five minutes, and dried with filter paper. With this method of staining the cytoplasm of the "bodies" is a magenta, light in the small bodies darker in the larger; the central bodies and chromatoid granules are a very dark blue, the nerve cell cytoplasm, a light blue, the nucleus a darker blue, and the red blood cells a brilliant eosin pink (Plate 18, Fig. 2). With more decolorization in the alcohol the "bodies" are not such a deep magenta and the difference in color between them and the red blood cells is not so marked.

The "bodies" and the structure are often more clearly defined with this method and perhaps on the whole it is better to use it for making diagnoses;† but when there

*Azur II—Eosin	3.0 g.
Azur II	0.8
Glycerin (Merk. chem. pure)	250.0 c.c.
Methyl alcohol (chem. pure)	250.0

Both glycerin and alcohol are heated to 60° C. The dyes are put into the alcohol and the glycerin is added slowly, stirring. The mixture is allowed to stand at room temperature over night, and after filtration is ready for use.

The solution is prepared ready for use by Grübler, Leipzig.

† Dr. Poor recommends it strongly for diagnostic purposes.

are only tiny "bodies" present, or when the brain tissue is old and soft, the Giemsa stain seems to be the more successful; above all, when one wishes to study the nature of the central structures and granules the Giemsa stain must be used. We therefore recommend strongly the use of both methods. Even if both are used and one has to wait for the longer method, the technic is far simpler than any so far published.*

Not only do the "bodies" come out more distinctly by the smear method, but the pathological changes accompanying them are well demonstrated. For instance, the swellings of the neuro-fibrils described by Ramon y Cajal, the collections of the lymphoid cells, the increase of the endothelioid cells, the degenerated nerve cells are all clearly seen.

The technic of the section work is as follows: (1) The small pieces are left in Zenker's fluid for three to four hours; (2) washed in tap water for five minutes; (3) placed in 80 per cent alcohol+iodine (enough tincture of iodine added to give port wine color) for about 24 hours; (4) 95 per cent alcohol+iodine 24 hours; (5) 95 per cent alcohol 24 hours; (6) absolute alcohol from four to six hours; (7) cedar oil until cleared; (8) cedar oil+paraffin 52° aa, two hours; (9) paraffin 52° two hours in each of two baths; (10) boxing; (11) sections are cut at 3 to 6 μ , dried in thermostat at 36° C. for about 24 hours protected from the dust, and stained according to the eosin and methylene blue method recommended by Mallory. The most important point in the technic is the time the material is allowed to remain in Zenker. According to our experience, two hours fixation is not enough, three to four hours is very good, and with every hour after five hours the results become less satisfactory. Left in Zenker over night the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, tiny forms found by us in sections from fixed virus. Another point in favor of the short fixation in Zenker is that the precipitate formed by the mercury is not so great and is more easily got rid of, which is a very great help in the identification of the tiniest forms. Schiffmann recommends short fixation in Zenker, but he does not state the time he finds best.

It is thought, also, that washing for any great length of time in water after fixation does not help the specimens, the few that were left for a much longer time than the five minutes are not as satisfactory as the others.

In regard to the rest of the technic, it is sufficient to say that the changes to the different fluids were made with great regularity, and the final differentiation in alcohol of the stained sections was done most carefully.

In the sections made in this way we have been able to demonstrate clearly very tiny forms as well as good structure in the larger forms, a description of which will be given in Part II.

* Van Dieson working in our laboratory, suggests a staining method which differentiates the "Negri bodies" more quickly than either of the two methods described above. So far, the best proportion of the stains used have not been determined, but satisfactory results have been obtained from the following mixture: To 10 drops of distilled water three drops of a sat. alc. sol. of rose anilin violet and six drops of Löffler's solution of methylene blue are added. The smears are fixed while moist in methyl alcohol for one minute. The stain is then poured on, warmed until it steams, poured off, and the smear is rinsed in water and allowed to dry.

The cytoplasm of the "bodies" is a deep and distinctive red, their inner structures are a dark blue, the nerve cells are a light blue and the blood cells a pale salmon-red.

The staining mixture remains good for about an hour.

TABLE I.
RESULTS OF EXAMINATION OF RABIES MATERIAL BY MEANS OF SMEARS, SECTIONS,
AND ANIMAL INOCULATIONS.

No.	Species	Date of Autopsy	Clinical Diagnosis	Presence of Negri Bodies in Smears	Presence of Negri Bodies in Sections	Result of Animal Inoculation	Presence of Negri Bodies in Smears from Animals Inoculated	Presence of Negri Bodies in Sections from Animals Inoculated
		1905						
1..	Dog	11-10	Rabies	+	..	+
2..	"	11-23	"	+	+
3..	"	12-2	Doubtful	+	+
4..	"	12-4	Rabies	+	+
5..	"	12-9	Suspicious	-	-	-
6..	"	12-9	"	-	-	-
7..	"	12-9	"	-	-	-
8..	"	12-15	Rabies	+	..	+	+	+
		1906						
9..	"	1-4	"	+	+	+	+	+
10..	"	1-10	"	+	+
11..	"	1-18	Doubtful	-	..	-
12..	"	1-22	Rabies	+	+	+	+	+
13..	"	1-26	"	+	+	+	+	+
14..	"	1-29	"	+	+
15..	"	2-20	"	+	+	+	+	..
16..	"	2-23	"	+	+	+	+	+
17..	"	2-26	"	D'tful*	+	+	+	+
18..	"	2-26	Doubtful	-	-	-
19..	"	2-27	Rabies	+	+	+
20..	"	3-2	"	+	+	+	+	+
21..	"	3-3	"	+	+	+	+	+
22..	"	3-6	Distemper or Rabies	+	+
23..	"	3-12	Rabies	+	+	+	+	+
24..	"	3-13	"	+	+	+	+	+
25..	"	3-26	"	+	+
		1905						
26..	Cat	12-5	"	+	..	+
27..	Human	11-10	"	+	+	+
28..	Child	11-16	"	+	+	+	+	..
		1906						
29..	"	1-16	"	+	+	+	+	+
30..	Human	1-9	Alcoholic neuritis	-	-	-
31..	Dog	1-4	Inoculated with human rabies. No symptoms	-
32..	"	1-16	Inoculated with human rabies. No symptoms	-	-
33..	"	1-30	Inoculated with human rabies. Typical symptoms	+	+
34..	"	1-31	Inoculated with human rabies. Typical symptoms	+	+	+
35..	"	2-6	Inoculated with human rabies. Typical symptoms	+	+	+
36..	"	2-15	Inoculated with human rabies. Typical symptoms	+	+	+	+	+
37..	"	3-6	Inoculated with street rabies. Typical symptoms	+	..	+	+	+
		1905						
38..	Calf	11-5	Normal	-
39..	Dog	11-13	"	-
40..	"	11-14	"	-
41..	"	11-14	"	-
42..	"	12-1	"	-
43..	"	12-1	"	-
44..	"		"	-	-	-

* Brain in bad condition. Two days old.

† A few tiny "bodies" found.

In Table 1 we have given the results of the animals studied with a view of determining the four points mentioned at the beginning of this section. In some of them the full examination as planned was carried out, in others, besides the smears, only sections or animal inoculations were made. The controls are not as many as we might have made had not so much control work been done previously by us and by so many others.

The results are as follows:

1. No control animal shows appearances similar to the "Negri bodies," either in smears or in sections. The various suspicious cases, especially the case of the dog with filaria, we consider among the best controls, because here we are dealing with animals dead after symptoms similar to those of hydrophobia.
2. In all of the cases proved by the biological test to be hydrophobia, "Negri bodies" are found in either smears or sections or in both.
3. In the animals which had been inoculated from these animals, "Negri bodies" are found in either smears or sections or in both.
4. The general characteristics of the "bodies" seen in the smears are similar to those of the "bodies" seen in sections.
5. The three tests correspond as to diagnostic results.
6. The smear method is much better than the section method in demonstrating the "bodies" for diagnostic purposes.
7. When the "bodies" are present in the smears the diagnosis of hydrophobia is certain, even if the biological test is negative. When they are not found the diagnosis is uncertain.
8. In a very few cases of street rabies, only extremely tiny forms are found. These may be easier to find in sections than in smears.
9. In doubtful or negative cases both the section method and animal inoculations should be tried.

II.

In studying the nature of these bodies many points have only been touched upon and others are still being investigated, but we believe that enough new knowledge has been gained to warrant this publication. The plan of this part of the work is as follows:

1. The comparison of the general characteristics of the "bodies"

<ol style="list-style-type: none"> a) Size b) Shape c) Number d) Site e) Structure 	$\left\{ \begin{array}{l} \text{in smears.} \\ \text{in sections.} \\ \text{in hanging-drop.} \end{array} \right.$ $\left\{ \begin{array}{l} \text{in different species of animals.} \\ \text{in different animals of same species.} \\ \text{in different parts of same animal.} \\ \text{in different stages of the disease.} \\ \text{in different numbers of passages.} \\ \text{after different modes of inoculation.} \end{array} \right.$
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2. Detailed characteristics of structure.
 - a) Cytoplasm.
 - b) Central bodies.
 - c) Chromatoid granules.
 - d) Different shapes.
 - e) Division forms

$\left\{ \begin{array}{l} \text{transverse.} \\ \text{longitudinal.} \\ \text{budding.} \end{array} \right.$
--
 - f) Conjugation forms.
 - g) Stages at which different forms appear.
3. Relation between the time the central nervous tissue becomes infected and the time the bodies appear.
4. Spread of the bodies to different parts of the host.
5. Significance of the bodies and comparison with known organisms.
6. Summary.

1. *General characteristics of bodies in smears compared with those in sections: Size.*—The majority of the forms seem larger in smears than they do in sections from the same case. The largest forms measured are about $18\ \mu$ and the smallest structured forms about $0.5\ \mu$. We can easily see that a form appearing as $0.5\ \mu$ in a smear might scarcely be visible in a section, and that such tiny forms, considering their extreme plasticity (see under structure), might easily pass the coarser Berkefeld filters. We have found that the size varies more with the course of the disease (which includes the question of accustoming the virus to the host, e. g. fixed virus), than it does merely with different species of animals. This means that the bodies may vary greatly in different animals of the same species in different parts of the same animal and at different stages of the disease. We may say in general that no very large forms are found in the early stages

of the disease or in any stage in certain varieties of especially susceptible animals to which the virus has become accustomed (fixed virus). While in later stages of the disease in animals inoculated with virus from another species, or in varieties of animals that are not fully susceptible to the disease, both large and small forms are found.

We have not yet had the opportunity of examining smears from rabid cattle, so we are not able to corroborate the statement of Negri that the largest forms are found in this variety of animal; but if it holds, it would seem that the reasons for the fact might be that cattle are among the less susceptible animals, and that they are generally inoculated with a virus from a different species of animal. Of course, other things being equal, we should expect a certain amount of variation in size and structure of an organism growing in different species of animals, just as we get variations in the same variety of bacteria and of other low forms of life grown in different culture media.

Shape.—The shape of the bodies appears more varied in smears than in sections, due partly to the fact that there is a certain amount of distortion. The distortion, however, is very slight, because within narrow limits of disturbance (i. e., too much or too unequal pressure in making the smears) the bodies are broken up and their identity lost. The principle types of shapes seen in smears are given in the accompanying plates. Plate 19, Figs. 3 to 56 inclusive, and the photographs may be studied in this connection. The same types of shapes are seen in all varieties of animals studied.

Number.—Generally more bodies are seen in smears than in sections from similar parts of the same case. Since we have learned to identify many tiny bodies, we have found that there are more in all cases, including fixed-virus cases, than have hitherto been reported. In any case we feel that we are able to demonstrate enough forms, or, at least, to account for enough forms, to correspond to the degree of infectivity of the part.

Site.—As is shown in Plate 18, Fig. 2 the topography of the bodies may be well preserved in smears. Their situation in the cytoplasm of the body and branches of the larger nerve cells is well shown. In parts of the smear which are more broken up the bodies may appear as if lying free, and it is these bodies, if the pressure has not been too great, that show the structure best. Such bodies have for the most part been

chosen for the photographs (especially 1, 2, 4, and 5). There are often many tiny "bodies" in degenerating nerve cells, but these show better in sections than in smears. The tiny forms which we have seen in the nuclei of the host cells also appear more distinct in sections than in smears.*

Structure.—The principal point in favor of the smear method of examination is that the structure of the bodies comes out so clearly and so characteristically that it is easy to draw a close analogy between it and that of known Protozoa. In the first place, as has been shown by Negri and most of the other investigators, the following fact holds true: Whatever the variety or species of animal infected, the bodies preserve their same general characteristic structure, i. e., a hyaline cytoplasm with an entire margin, and with one or more inner bodies having a more or less complicated and regular structure. This fact alone, that by such an entirely different method of examination the bodies show the same characteristic structure in so many different varieties of animals, is a very strong point in favor of their not being degeneration forms.

In general we may say the same things in regard to the relation between structure of the "bodies" and the variety, etc., of the animal, that we did when discussing size, because their structure varies to a certain extent with their size. The tiny forms, rounded, with a more or less centrally-situated chromatin-staining granule, slightly larger forms with three to several such granules (often four), elongated forms with a central chromatin line, and tiny forms in two or in groups of three or more (Plate 19, Figs. 3-8) are the only types found in fixed virus (with an occasional slightly larger form containing a larger central body and a few tiny granules). The tiny forms found in fixed virus seem to be far more delicate than apparently equally tiny forms seem in other lesions, that is, they take the stain more delicately, the central structure is not so distinct, and the whole body is more easily destroyed by pressure in the former than in the latter case. Hence, it is only in the best made smears that these fixed-virus forms are seen, and then only after the eye has been accustomed to their very delicate coloring and outline.

The forms found in fixed virus animals are the only ones which

* With van Gieson's new staining method these tiny forms are well differentiated in smears.

are better preserved or at least which are more distinctly seen in sections than in smears. This is due probably to their extreme delicacy. The fact that we have found very many forms in all cases (15) of developed fixed-virus infection studied makes it probable that they are present in every case and that they come out better with the technic described in Part I than with the technic followed by other investigators. In regard to their specificity, we would say that we have made few controls for the following reason. As slight alterations in technic seem to interfere with their demonstration, and as, therefore, their non-appearance might not mean that they are not present, large numbers of animals would have to be examined before one could be sure that forms simulating them might not be present in certain cases. The facts, however, that in our four controls and in the first two days after inoculation of a series of ten experimental rabbits (see below for details of this experiment), they are not found, and that when they do appear they possess certain characteristics, in structure, site, and number corresponding to the course of the disease, makes it pretty evident that we are dealing with the specific organism. These bodies have the following characteristics: They are tiny rounded forms, sometimes wavy in outline, as if possessing slight amœboid motion, sometimes elongated, extending along the rim of the host-cell nucleus, or along one of the nerve fibrils, as if moving there; they take a delicate light magenta stain very similar to that taken by the small serum globules in the blood vessels, and it would be difficult, if not impossible, to distinguish some of them from these serum globules, if they were in the blood vessels. Many of the organisms, however, show a small chromatin granule, situated more or less eccentrically, sometimes on the very rim of the body. In the larger forms the granule is large, in the smaller it cannot always be seen (Plate 18, Fig. 1); some of the larger forms show from two to several granules and occasionally there is a body with the definite central body and the small granules about it. In these fixed-virus sections we have found certain tiny bodies in some of the nerve-cell nuclei, especially in the smaller of those cells which show decided degenerative changes of the cytoplasm. These intranuclear forms seem to stand out quite distinctly from the rounded, acid-staining degenerative masses. The latter are not so refractive as the former. The intranuclear forms have not yet been studied suffi

ciently to allow a decided opinion in regard to their place in the life-history of the organism. They are quite frequent in the olfactory bulbs of guinea-pigs after inoculation with rabbit-fixed virus.

The fact that none of the larger forms of the "bodies" are found in animals dying after fixed-virus inoculations is an added indication that the bodies are not products of degeneration of the host cells.

That the development of only these tiny forms with their simple structure in fixed-virus animals is due to the fact that the special strain inoculated is accustomed to the one variety of host is shown by the result obtained by inoculating the strain into another variety of animal. We have inoculated one dog and several guinea-pigs subdurally, and three mice subcutaneously with fixed virus from the rabbit, and in each case (in only one case in mice, as only one of the three died) besides the tiny forms there have been numerous large forms with the characteristic, definite, more or less complicated structure (corresponding to Plate 19, Figs. 17-34). This is contrary to the results obtained by Schiffmann upon inoculating rabbit-fixed virus into dogs. In his cases he could find no bodies whatever. On the other hand, we have had delayed fixed-virus action in one rabbit (inoculated with 2 c.c. of a thin emulsion into the ear vein, with death on the 11th day after typical symptoms of paralytic rabies), and in this animal we found only the tiny, delicate forms found in the other fixed-virus rabbits.

In regard to variations in structure at different stages of the disease, most of our study has been made upon animals inoculated with fixed virus, and the forms and structure in these cases seem to be about the same in the early stages as in later ones. It would seem that under these favorable conditions for the organism it grows and divides so rapidly from the beginning, and infects so many of the host cells that the animal is overwhelmed before the parasite has a chance to develop the larger forms. The results are different in the animals inoculated with street virus.

We inoculated one series of seven rabbits with street virus from a dog, killed the first animal on the seventh day after, and the others respectively on the 9th, 11th, 12th, 14th, 16th, and 17th days. The results as to number and structure of the bodies are briefly as follows:

Seventh-day rabbit.—In the bodies of the large nerve cell of Ammon's horn and cerebral cortex an occasional tiny form and an occa-

sional one of the intermediate grades were seen. (Forms corresponding to Plate 19, Figs. 3-16.)

No definite extracellular forms were seen, but neither sections nor smears have yet been studied minutely. This is the earliest day reported for forms found after inoculations with street virus. Negri reports finding them on the 10th day in a dog. In our series of animals those that were allowed to remain alive did not begin to have visible symptoms until the 13th and 14th days.

Ninth day rabbit.—Many very definitely structured forms were seen in the large nerve cells of practically all parts of the cerebral nervous system, smears and sections showing equally well. The forms corresponding to Plate 19, Figs. 3-12 were in the majority, those corresponding to Figs. 13-16 in moderate numbers, and those corresponding to Figs. 17-32 occasionally.

Eleventh-day rabbit.—Practically no difference between it and 9th day one.

In the 12th, 14th, 16th, and 17th day rabbits the larger forms appeared in gradually larger numbers and many more division forms were seen.

So far most of the study in this series has been made on the earlier stages.

There are no marked differences in the "bodies" found in different parts of the central nervous system of one animal dead 25 days after inoculation into the sciatic nerve. The general histological lesions are more intense in the cord and there is a larger number of the larger "bodies" there than usual, but the "bodies" in the brain are about the same in number and structure as in animals dying from subdural inoculations.

Appearance of "bodies" in hanging drop.—So far, we have done only enough work with the hanging drop to make us realize that it is an extremely difficult method of study and needs most careful control at each step. There is no doubt that certain forms of the organism can be recognized; but the nerve tissue elements change so quickly, assuming flagellated and delicately granular form which simulate those of known organisms that the control must be at one's side before one realizes that the object studied is not a living organism.

Detailed characteristics of structure.—In smears as well as in sections, the *cytoplasm* appears quite homogeneous, there is no evidence of a reticulum, or of a granular structure outside of the definite chromatoid granules. The smears, however, have brought out one important point in regard to the cytoplasm more clearly than the sections, and that is that it is more basophilic than acidophilic in staining qualities. With the Giemsa stain, as we have seen in Part I, it takes the methylene-blue stain more than the eosin-red, and even with the simple eosin methylene-blue stain the protoplasm appears as a deep magenta unless much decolorized.

One of the points, then, which has been brought up against the protozoan theory falls to the ground. The cytoplasm takes the stain as does that of many well-known protozoa—the malarial organism, for instance.

In studying the *central bodies* of these organisms, as they appear in the smears, one of the first things noticeable is that they are not surrounded by a clear space—that there is no sign of a vacuolar appearance in the whole body. This is a very different appearance from that given in the sections, and it shows that the vacuoles described in the sections are artefacts due to the technic. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The general type of the structure of the central body is that of well-known protozoan nuclei; for example, Prowazek gives a description of the nucleus in certain stages of the *Plasmodiophora brassicae*, which might be used here to describe the most typical appearance of these central bodies.

The chromatin is arranged in a more or less granular ring around the periphery of the central body or nucleus leaving an achromatic or more acid-staining center in which is situated, generally eccentrically, a varying-sized karyosome (Plate 19, Fig. 37). There are a number of variations from this principal type, according to stage of development. Often the whole nucleus answers to the description of the compound karyosome given by Calkins in his description of the protozoan nucleus. In the tiny “bodies” the chromatin can only be seen as a dot, in those a little larger it may be a large solidly staining gran-

ule, or a ring or rod, the latter often hour-glass shaped. In forms large enough for the characteristic structure to be developed and to be clearly seen, the central body may show evidence of fragmentation (Plate 19, Figs. 18, 38, 51, etc.). Just such evidence of fragmentation is shown in many protozoan nuclei preparatory to division. It is interesting that forms showing this phase, and, moreover, very similar in general appearance to some of the forms seen here, have been depicted by Doflein in the early stages of the life-cycle of *Glugea lophii*, a myxosporidium, parasitic in the ganglion cells of a fish (*Lophius piscatorius*).^{*} The staining of the nucleus will be considered with that of the chromatoid granules.

The chromatoid granules are most frequently arranged in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to active changes of growth and division. They take generally a more mixed chromatin stain than the chromatin of the nucleus. This fact is brought out in the Giemsa-stained smears. Here the nuclear chromatin takes generally a definite azure tint, while the chromatoid granules are more of a blue, though sometimes they may appear more red. That the red in the central body and granules is not an eosin-red, is shown first by its peculiar magenta tint, and second by the fact that when partly decolorized by methyl alcohol, the red color disappears from these structures leaving them a dark blue, while the cytoplasm is a pale blue-pink and the red blood cells are a definite eosin-pink. If a dilute methyl alcohol is used, an interesting series of differentiations in color may be obtained. Such a more or less regular arrangement of chromatoid granules in the cytoplasm of Protozoa is of frequent occurrence (Calkins, Minchin). It is a marked feature, according to the observations of one of us, in certain stages of the *Plasmodiophora brassicae*. The further changes in the central bodies and granules will be considered under division forms.

Different shapes.—We agree with Negri in considering many of the different shapes due to the position of the organism in the host cell. There is no doubt that the substance of these bodies is extremely delicate and plastic, easily adapting itself to the position in which it

^{*} In Doflein's later classification (1901) he names this species *Nosema lophii* and places in the sub-order *Microsporidia* under the order *Cnidosporidai*.

is found and easily destroyed by artificial means. Many of the elongated forms are forms growing and dividing in this way because of position between the fibrils. The triangular forms (Plate 19, Figs. 26, 50, and Plate 21, Fig. 7) are probably forms that have grown in the angle made by the giving off of a nerve cell branch. They have been placed by us, in Plate 19, underneath the much elongated forms as possible division forms of the latter; but they probably are not. The principal cause of most of the different shapes, however, is the rapid growth and division of the organism.

Division forms.—The whole picture is one of rapid growth and multiplication, and this corresponds with the clinical history. The elongated forms containing from two to five or even six nuclei are the result of rapid nuclear division without corresponding cell division. This condition is found quite frequently in Protozoa (*Thelohania Mulleri*, Minchin, p. 292). The elongation in this way is probably due, as we have said, to the position of these bodies between the nerve fibrils, and to their great plasticity.

Under the most favorable conditions (fixed virus), growth and division occur most rapidly and simply, the tiny forms dividing and redividing apparently indefinitely. Whether there is simple conjugation, or fusion of unequally divided forms during this condition, it is difficult to say. It would probably take much study to settle this question. Small mulberry masses are found during this stage, but whether they are the result of the breaking up of a larger form or of the rapid division of a tiny form it is impossible for us to say as yet. We have also seen appearances which suggest plasmodial phases. There seems to be distinct evidence of an intranuclear invasion also in fixed-virus infection.

In cases where there has been an inoculation of comparatively small quantities of the virus, i. e., a small number of forms of the parasite capable of immediate infection, or in cases where there has been an infection of less susceptible animals (dogs, cattle, human beings, etc.), or with a less accustomed virus (fixed virus of rabbits into guinea-pigs or mice), we get a slower growth with its larger structures and different division forms. The chromatin accumulation in the form of a definite nucleus, apparently undergoes fragmentation very easily, and so we have forms containing two to several central bodies,

some rounded (Plate 19, Figs. 12, 13, 14, 19, etc.), some elongated (Fig. 15), some of unequal division, similar to budding (Fig. 29). Then we find forms with bodies apparently differentiated within one membrane (Figs. 20, 31, 53), and bodies with practically all stages of hour-glass constriction, indicating transverse division (Fig. 32). Many pairs, unequal in size, apparently fusing or dividing have been seen (Figs. 33, 45), and finally, we have large bodies with the chromatin scattered throughout the whole organism in the form of tiny, unevenly rounded or elongated masses, one or two larger, indicating the remains of the nucleus, and in these forms we get all stages of apparent budding (Figs. 40, 41, 42, 54, 55). The buds vary somewhat in size, some being very tiny. The formation of buds accounts for the appearance in the same cell of both large and small forms. It also helps to account for the rapid spread of the organisms. These tiny budded forms similar to "swarm spores" are probably motile and pass quickly to other host cells.

We have also found a number of more or less indefinite masses, taking the stain a little more deeply than the other bodies, and apparently made up of large numbers of tiny bodies, but so far they have been too indefinite for us to be sure that we have cystlike structures. We have not studied the sections minutely enough yet to find out how such structures appear there, or whether they are similar to the "cysts" described by Negri.

Conjugation forms.—At first sight "the buds" were thought by us to be possibly conjugating individuals, but when on further study they were found to be principally, if not entirely, in forms which showed marked fragmentation of the chromatin, they were interpreted as budding forms. Such unequal forms as are represented in Plate 19, Figs. 33 and 45 may be conjugating forms, but so far we have not been able to decide as to their significance.

The relation between the time the central nervous tissue becomes infective, and the time the bodies appear.—Our principal work on this point has been done with fixed virus. After finding that tiny, characteristic forms were found in two rabbits dying on the eighth and ninth days after subdural inoculation with fixed virus, we inoculated 10 rabbits subdurally with fixed virus (629th passage), killed one every day by chloroform, and examined the central nervous system in the

following way: One-half of the brain and medulla, including the olfactory bulb, was cut into slices, and with slices from the dorsal and lumbar spinal cord, including one or two spinal ganglia, was placed in Zenker, and subjected to the technic for sections mentioned in Part I. From the other half of the brain, and corresponding parts of the cord, two sets of smears were made, and each stained respectively by the two methods mentioned in Part I. Unfortunately, with this series of animals, we did not test the virulence of the nerve tissues, so we do not know at exactly what period it became distinctly virulent. However, in an earlier series of eight rabbits inoculated in the same way, and from which only smears were made, Dr. Poor tested the virulence roughly, as follows: One animal was killed each day, with the exception of the eighth, which died on the ninth day. From the lumbar cord, and from Ammon's horn, pieces of about the same size, so far as we could judge from eye measurement, were cut. Two dilutions were made from each piece, a stronger one, by the addition of 3 c.c. of normal salt solution, making an emulsion; and a weaker one, by making a 1:1,000 dilution of the stronger. Two guinea-pigs were inoculated with the weak dilution $\frac{1}{2}$ c.c. each; two with the strong dilution, $\frac{1}{2}$ c.c. each.

Of the animals inoculated with the weak dilutions of the *cord*, none died; of those inoculated with weak dilutions of the *brain*, none died from the first or second day rabbits, one died from the third day, and one from the fourth day animal, none from the fifth day, one from the sixth day, two from the seventh day, and none from the ninth day animal. Of the animals inoculated with the strong dilutions of the *cord*, none died from first, second, third, and fourth day rabbits; one from the fifth day, one from the sixth day, and none from the seventh day animal. Eighth and ninth day animals were not inoculated. Of the animals inoculated with the strong dilutions of the *brain*, none died from first and second day rabbits, two died from the third, fourth, fifth, sixth, and seventh day rabbits, eighth and ninth day animals not inoculated.

In this experiment, then, the weak dilution of the *cord* was not infective in the doses used; the strong dilution was not infective until the fifth day, and then not regularly so; while both dilutions of the *brain* became infective on the third day, the weaker one less so, and

continued so to the end. These results corroborate the work of Remlinger, who found the medulla virulent on the third or fourth day after subdural inoculations of fixed virus.

In neither of these sets of experiments has the approximate number of organisms present been shown, and until we know this we cannot say that in any measured amount of infective material there may be more than an occasional tiny form, which it might be very difficult, perhaps impossible, to find in sections or smears of such material.

In the examination of the 10 rabbits mentioned first in this connection, although we have so far studied only a comparatively few sections, we have found the bodies appearing as follows: On the first, and second days none; on the third day an occasional one in the large lymphoid cells of the perivascular lymph spaces at the base of Ammon's horn; on the fourth day, a few tiny undoubted ones in the large nerve cells of the olfactory bulb, of the lower curve of Ammon's horn, and of the motor area of the cerebral cortex; on the fifth, a moderate number in the same areas and in scattered cells throughout the whole brain; on the sixth, many in the same areas, and in the medulla; on the seventh (two animals), on the eighth, and on the ninth, very many, as in the other fixed-virus animals studied (Plate 18, Fig. 1).

From this series of experiments it seems that the bodies may be found soon enough and in practically large enough numbers to account for the beginning infectivity of the nerve tissue, and that with only a little more careful experimenting this may be brought out clearly.

Four control rabbits were studied in this connection; two normal rabbits, one which had died from pneumococcus infection, and one from yeast infection.

Spread of the bodies to different parts of the host.—This point is now being studied by us. It is taken up under two heads; first, the spread of the organisms from the point of inoculation, and second, its spread from the site of infection.

In whatever way the virus enters the body, so far as we know, there is no development of the organism, or none, to any appreciable extent, until it reaches the central nervous system, and not until after a certain amount of development there does it infect the peripheral organs. Before the disease was well studied it was thought that the salivary glands were the chief site of the infection. But it has been shown

that these glands are not always infective, and when they are, not until comparatively late in the disease and that when the virus is inoculated into them, the animal seldom comes down with the disease and probably never if the centripetal nerves are cut (Bertarelli). This means that the parasite does not grow in the salivary glands, that it is only carried there incidentally by its spread from the central nervous system along the nerve branches. That the organisms escape into the blood and are carried in this way in small numbers is shown by the fact that the blood in large quantities has been found infective (Marie). Principally by the nerve channels, secondarily by the blood and lymph channels, the organisms are carried in small numbers to all parts of the body. With other investigators, we have found the suprarenal capsules infective (in one out of two street-rabies dogs). One of the three guinea-pigs inoculated died after typical symptoms of rabies, and the central nervous system showed many good-sized bodies and was infective for other animals. If it is true that the organisms pass in such comparatively small numbers to the various peripheral organs, and especially if only the smaller forms pass, then our chances of identifying them in the salivary and other glands are very slight. Smears from these parts are unsatisfactory, and we have not yet been able to study the sections.

In regard to the spread of the organisms from the point of inoculation, the parasites are probably carried to the central nervous system along channels similar to those by which they are carried away, and unless enough of them can quickly reach the nerve cells, they are probably destroyed by the macrophages. We have found, as we have said, what appear to be tiny bodies in the large lymph cells on the third day after inoculation with fixed virus. In one fixed-virus rabbit, found dead on the morning of the seventh day after inoculation, an animal which had been used before, and whose resistance was probably lessened, the central nervous system was loaded with large lymphoid cells many of which were apparently filled with tiny organisms. This question is still being studied.

Significance of the "bodies" and comparison with known organisms.—Although it may be questioned whether enough forms have been found to account for every stage in a life-cycle, it is certain that the great majority of the bodies stand out so clearly as organisms with such

definite, constant, characteristic structure and staining reactions and show so many forms similar to division forms of known Protozoa, that the picture is difficult to explain in any other way than as that of a developing organism belonging to the group Protozoa. It seems unnecessary further to consider the possibility of their being changed red blood cells or any other form of degeneration of the host tissue; and this alone is evidence in favor of their being organisms.

From time to time cases have occurred in which the "bodies" are seen in such numbers and in such stages of development that we are as sure of their being organisms as we are that the bodies photographed by Wright from Delhi boil, are organisms. As we study the picture further and find at almost every step analogies in the life-cycle of known Protozoa, the evidence is so overwhelming that there seems no reason to doubt that they are living organisms; the small single forms with their tiny chromatin central bodies rounded, elongated, or in twos and more, as in *Nosema lophii* and other Microsporidia (Doflein); the groups of small forms in twos and more (multiplicative reproduction of Doflein); the appearances of the central body in the larger forms similar to that of many protozoan nuclei at corresponding stages of development (Calkins, Prowazek); the many evidences of division of these larger forms such as fragmentation of the nucleus (Calkins), two nuclei, all stages in hour-glass constriction of the body; and finally, the distribution of the nuclear material throughout the whole organism with evidences of its fragmentation and of budding, a phenomenon which has been described as occurring in all classes of Protozoa (Calkins, Minchin)—all these and more make a collection of evidence which amounts to proof.

The parasite seems to possess more points of resemblance to organisms belonging to the sub-order Microsporidia, than to those of any other order.

SUMMARY AND CONCLUSIONS.

1. The smear method of examining the Negri bodies is superior to any other method so far published for the following reasons: (a) It is simpler, shorter, and less expensive; (b) The Negri bodies appear much more distinct and characteristic. For this reason and the preceding one, its value in diagnostic work is great; (c) The

minute structure of the Negri bodies can be demonstrated more clearly; (d) Characteristic staining reactions are brought out.

2. The Negri bodies as shown by the smears as well as by the sections are specific to hydrophobia.

3. Numerous "bodies" are found in fixed virus.

4. "Bodies" are found before the beginning of visible symptoms—i. e., on the fourth day in fixed virus, on the seventh day in street virus, and evidence is given that they may be found early enough to account for the appearance of infectivity in the host tissues.

5. Forms similar in structure and staining qualities to the others, but just within the limits of visible structure at (1,500 diam. magnification) have been seen. Such tiny forms, considering the evidence they give of plasticity, might be able to pass the coarser Berkefeld filters.

6. The Negri bodies are organisms belonging to the class Protozoa. The reasons for this conclusion are: (a) They have a definite, characteristic morphology; (b) This morphology is constantly cyclic, i. e., certain forms always predominate in certain stages of the disease, and a definite series of forms indicating growth and multiplication can be demonstrated; (c) The structure and staining qualities as shown especially by the smear method of examination resemble that of certain known Protozoa, notably of those belonging to the sub-order Microsporidia.

7. The proof that the "Negri bodies" are living organisms is sufficient proof that they are the cause of hydrophobia; a single variety of living organisms found in such large numbers in every case of a disease, and only in that disease, appearing at the time the host tissue becomes infective in regions that are infective, and increasing in these infective areas with the course of the disease can be no other, according to our present views, than the cause of that disease.

LITERATURE.

- ABBA, F., ET BORMANS, A. "Sur le diagnostic histologique de la rage," *Ann. de l'Inst. Past.*, 1905, 18, p. 49.
- BAILEY, F. R. "Studies on the Morphology of Ganglion Cells in the Rabbit," *Jour. Exper. Med.*, 1901, 5, p. 549.
- BANDINI, P. "Contributo allo conoscenza dei corpi di Negri nella rabbia," *Arch. per le sc. med.*, 1904, 15, p. 207.
- BERTARELLI, E. "Sui rapporti tra le modificazioni di virulenza del virus rabbico e le modificazioni dei corpi di Negri," *Riv. d. ig. e san. pubbl.*, 14, p. 790.

- . "Ricerche ed. osservazioni spermentale sulla rabbia" (Nota I, II e III), *ibid.*, 1903, 1904, 1905, 14, 15, 16. Published also in *Centralbl. j. Bakt.*, orig. 1904 and 1905.
- . "Die Negri'schen Körperchen im Nervensystem der wutranken Tiere, etc." *Centralbl. j. Bakt.*, 1906, 37, p. 556.
- E VOLPINO. "Osservazioni morfologiche e biologiche su un caso di rabbia umana con speciale riguardo ai corpi di Negri," *ibid.*, 1903, 14, p. 659.
- BOHNE. "Beitrag zur diagnostischen Verwerthbarkeit der Negri'schen Körperchen," *Ztsch. j. Hyg.*, 1905, 52, p. 87.
- BONGIOVANNI, A. "I corpi di Negri e l'infezione rabida da virus fisso a lento decorso," *Riforma medica*, 1905, 42, p. 1149.
- BOSC, F. J. "Recherches sur l'étiologie de la rage," *Compt. rend. d. l. Soc. d. Biol.*, 1903, 55, 1436.
- CALKINS, G. N. "The Life History of Cytoryetes Variolæ Guarnieri," *Jour. Med. Res.*, 1904, 11, p. 136.
- . "The Protozoan Nucleus," *Archiv j. Protistenkunde*, 1903, 2, p. 213.
- CELLI, A. "Res. della seconda reunione della società italiana di Patologia," *Lo sperimentale*, 1903, 57, p. 772.
- U. DE BLASI, D. "Ist das Wuthgift filtrirbar?" *Deutsche med. Wehnschr.*, 1903, 29, p. 945.
- COURMONT ET NICOLAS. "Étude sur la virulence de l'humeur aqueuse de lapins morts de rage," *Comp. rend. d. l. Soc. d. Biol.*, 1903, 55, p. 1595.
- DADDI, G. "Sull' etiologia dell' idrefobia," *Rib. crit. di clin. med.*, 1903, 4, 337.
- . "Ricerche sulla rabbia," *ibid.*, 1904, 5, pp. 334, 349.
- D'AMATO, L. "Sull' etiologia della rabbia," *Gazz. degli ospedali*, 1903.
- . "I corpi di Negri in rapporto all' etiologia ed alla diagnosi della rabbia," *Riforma med.*, 1904, 20, p. 617.
- . "Ulteriori ricerche sui corpi di Negri," *ibid.*, 1904, 20.
- DI VESTEA, A. "Nota micrografica sulla rabbia sperimentale del coniglio," *Atti d. r. Accad. med.-chir. di Napoli*, 1894, 47, N. S. 4.
- . "Dei più recenti studii circa la natura del virus rabico," *Gior. ital. d. Spc. med.*, Pisa, 1903, 1, p. 98.
- . "Ulteriori osservazioni circa la filtrabilità del virus rabido," *Med. italiana*, 1904, 2, p. 241.
- . "Di alcune proprietà biologiche dei filtrati rabici in confronto con le emulsioni di tessuto nervoso da cui provengono," *Riv. d' ig. e san. pubb.*, 1905, 16.
- DOFLEIN, F. "Studien zur Naturgeschichte der Protozoen. III. Über Myxosporidien," *Zool. Jahrb. (anat.)*, 1898, 5, p. 281.
- . "Die Protozoen als Parasiten und Krankheitserreger," Jena, 1901.
- DOMINICI, L. "Sul valore della diagnosi istologia nella rabbia," *Policlinico*, 1904, 11, p. 895.
- EWING, J. "Studies on Ganglion Cells," *Ar. h. of Neurology and Psychopath.*, 1900, 3, p. 263.
- FASOLI, G. "Sulla colorazione dei corpi di Negri nella infezione rabida," *Policlinico*, 1904, 11, p. 334.
- FROTHINGHAM, L. "The Rapid Diagnosis of Rabies," *Jour. Med. Res.*, 1906, p. 471.
- GALLI, VALERIO, B. "Recherches experimentales sur la rage des rats, etc." *Centralbl. j. Bakt.*, 1, 1905, 40, pp. 197, 318.

- GUARNIERI, G. "Ricerche sull' etiologia e della patogenesi della rabbia," *Clin. med.*, 1903, 9, p. 159.
- LUZZANTI, L. "Sulla diagnosi della rabbia," *Arch. per le sc. med.*, 1904, p. 521.
- . "La dimostrazione del parassita specifico in un caso di rabbia nell' uomo," *ibid.*, 1904, p. 167.
- . "Zur Diagnose der Tollwuth," *Ztschr. f. Hyg.*, 1905, p. 305.
- E MACCHI. "Sulla diagnosi della rabbia," *Gazz. med. italiana*, 1904, 55, p. 241.
- MAAS. "Ein Fall von Lyssa humana," *Münch. med. Wchnschr.*, 1905, 52, p. 119.
- MACNEAL, W. J. The Life History of *Tr. Lewisi* and *Tr. Brucei*," *Jour. Infect. Dis.*, 1904, 1, p. 518.
- MALLORY, F. B. "Scarlet Fever, Protozoon-like Bodies Found in Four Cases," *Jour. Med. Res.*, 1904, p. 483.
- MARESCCH, R. "Über die feinere Struktur der Negri'schen Körperchen," *Wien. klin. Wchnschr.*, 1905, 25, p. 659.
- MARIE, A. "La virulence du sang chez les animaux rabiques," *Comp. rend. d. l. Soc. d. Biol.*, 1905, p. 544.
- MARTINOTTI, C. Alcune osservazioni e considerazioni su cervelli e gangli spinali di conigli morti per infezione di virus rabico," *Giorn. d. r. Accad. di med. di Torino*, 1903, 4 s., 9, p. 355.
- MARZOCCHI, V. "Contributo alla questione della specificità dei corpi di Negri," *Arch. per le sc. med.*, 1904, p. 85.
- MINCHIN, E. A. See *Myxosporidia*. In M. Ray Lankerster's "Treatise on Zoölogy," First Edition, London, 1903, p. 274.
- NEGRI, A. A series of articles on the "Etiology of Hydrophobia," beginning March 27, 1903, and all except two articles published in Italian Journals. The two articles came out in the *Ztschr. f. Hyg.*, and sum up the work published up to that time in Italy. (The principal articles are given below.)
- . "Contributo allo studio dell' eziologia della rabbia," *Boll. d. Soc. med.-chir. di Pavia*, 1903, p. 88.
- . "Sull' eziologia della rabbia. La dimostrazione del parassita specifica nell' infezione rabica degli uccelli," *ibid.*, 1904.
- . "Sull' eziologia della rabbia; Sulla morfologia e sull' ciclo evolutivo del parassita specifico," *ibid.*, 1905, N. 3, p. 321.
- . "I risultati delle nuove ricerche sull' eziologia della rabbia," *Lo sperimentale*, 1904, 58, p. 273.
- . "Beitrag zum Studium der Aetiologie der Tollwuth," *Ztschr. f. Hyg.*, 1903, 43, p. 507.
- . "Zur Aetiologie der Tollwuth: Die Diagnose der Tollwuth auf Grund der neuen Befunde," *ibid.*, 1903, 44, p. 520.
- PACE, D. "Osservazione e ricerche sulla rabbia," *Atti del Congresso di med. intern.*, Padova, 1903.
- . "I corpi di Negri nel systema nervosa dell' uomo idrofobo," *ibid.*, 1903.
- . "Sopra alcune speciale formazione eosinofile simulanti i corpi di Negri nelle cellule dei gangli cerebro spinali dell' uomo idrofobo," *Riforma med.*, 1904, 20, p. 673.
- . "Prime ricerche sperimentale e microscopiche sulla rabbia aviare," *Congresso della Società italiana di med.*, Roma, 1904, 24/27 Ott.

- POOR, D. W. "Pathological Studies in Rabies," *Proc. N. Y. Path. Soc.*, 1904, p. 101.
- . "Recent Studies in the Diagnosis of Rabies," *Med. Rec.*, 1905.
- PROWAZEK, S. "Über den Erreger der Kohlhernie, Plasmodiophora brassicae," etc., *Arbeit a. d. kaiserl. Gesundheitsamte*, 1905, p. 396.
- RAMON Y CAJAL, S., AND DAHMACEO-GARCEA, D. "Las lesiones del retículo de las células nervosas su la rabia," *Trabajos del Laboratores de investigaciones biológicas de la Universidad de Madrid*, 1904, 3, p. 273. (*Ref. Bull. de l'Inst. Past.*, 1905, 7, p. 298.)
- REMLINGER, M. P. "Insolement du virus rabique par filtration," *Comp. rend. d. l. Soc. de Biol.*, 1903.
- . "Le passage du virus rabique à travers les filtres," *Ann. de l'Inst. Past.*, 1903, p. 834; and 1904, p. 150.
- . "A quel moment le bulbe des lapins rabiques de passage durent il virulent?" *Comp. rend. d. l. Soc. de Biol.*, 1905, pp. 815 and 973.
- SCHIFFMAN, J. "Zur Kenntniss der Negri'schen Körperchen," *Wien. klin. Wchnshr.*, 1905, p. 657.
- . "Zur Kenntniss der Negri'schen Tollwuthkörperchen," *Ztschr. f. Hyg.*, 1906, 52, p. 199.
- SCHUDER. "Der Negri'sche Erreger der Tollwuth," *Deutsche med. Wchnschr.*, 1903, p. 700.
- STANTON, E. MACD. "The Etiology of Rabies," *Albany Med. Jour.*, 1905, p. 184.
- "Travaux récents de la rage," *Bull. de l'Inst. Past.*, 1905, p. 753.
- VOLPINO, G. "Sopra alcuni reperti morfologici nelle cellule nervose di animali affetti da rabbia sperimentale," *Giorn. d. r. Accad. d. med.*, Torino, 14, 1903, p. 228.
- . "Sulla diagnosi istologica della rabbia," *Riv. d. ig. e san. pubb.*, 1904, 15, p. 20.
- . "Sulla struttura dei corpi descritti di Negri nella rabbia," *Arch. per le sc. med.*, 1904, 28, p. 153.
- . "Sulla struttura dei corpuscoli contenuti nell'interno dei corpi di Negri," *Riv. d. ig. e san. pubb.*, 1905.
- . "Über die Bedeutung die dem Negri'schen Körperchen, etc.," *Centralbl. f. Bakt.*, 1905, 37, p. 459.
- WAY, C. "The Negri bodies and the Diagnosis of Rabies," *Am. Vet. Rev.*, 1905, 29, p. 937.
- WILLIAMS, A. W. "Negri Bodies, with Special Reference to Diagnosis," *Proc. N. Y. Pathol. Soc.*, 1906, N. S. 5, p. 155.
- WILSON, R. J. *Proc. N. Y. Pathol. Soc.*, 1897 and 1898, p. 233.
- WRIGHT, J. H. "Protozoa in a Case of Tropical Ulcer ('Delhi Sore')," *Jour. Med. Res.*, 1903, 10, p. 472.

DESCRIPTION OF PLATES 18 AND 19.

All figures are drawn from the object and are equally magnified (about 1,200 diameters). Objective, Zeiss apochromatic 16 \times millimeter. Apert. 130; Ocular, Zeiss compensating 6; drawn-tube 16.

Abbreviations:

N.—Nucleus of nerve cell
br.—Branch of nerve cell
D.—Degenerated nerve cell
bv.—Blood vessel
rb.—Red blood cell

B.—"Negri body"
C.—Central body of "Negri body"
K.—Karyosome
ch.—Chromatoid granules

FIG. 1.—Section (about 5 microns) through large pyramidal cells of Ammon's horn of rabbit which was chloroformed while dying on *eighth* day after subdural inoculation of fixed virus (426th passage). Fixed in Zenker, and stained with eosin and methylene blue, according to the method described in text. In the cytoplasm of nearly every large nerve cell is situated one (sometimes two or three) tiny "Negri body," which is often near the nucleus and is sometimes elongated, extending along the nuclear membrane. These tiny bodies take a faint magenta-pink stain in contrast to the red blood cells, which stain a brilliant eosin-red, and most of them show within their substance a minute dark blue granule situated more or less eccentrically; sometimes at the extreme periphery of the body. In some of the smaller bodies these granules cannot be seen, probably on account of their extreme minuteness.

FIG. 2.—Smear from Ammon's horn of dog chloroformed while dying on 20th day after subdural inoculation with human rabies. Fixed in Zenker's fluid and stained with eosin and methylene blue after the method described in text.

Both bodies and branches of some of the large, blue-staining nerve cells show quite distinctly, while others are more or less destroyed. Definitely within the bodies and branches of some of these nerve cells and lying in the neighborhood of others, are seen the magenta-staining "Negri bodies" of various sizes and shapes with their central "bodies" and "chromatoid granules" staining a dark blue. The small blood vessel lying across the center shows the red blood cells staining an eosin red.

FIGS. 3 to 56, inclusive.—Some of the forms of "Negri bodies" seen in smears stained with Giemsa's solution, and arranged according to possible modes of growth and multiplication. The cytoplasm of each body stains a homogeneous pinkish blue, darker in the larger forms, very light in the tiny ones, the central body and the chromatoid granules stain a bluish red, the latter generally more blue than the former.

FIG. 3.—Tiniest structured form seen.

FIG. 4.—Elongated central body.

FIG. 5.—Two central bodies.

FIGS. 6, 7, and 8.—Apparent division forms of 5.

FIG. 9.—Form containing a larger, solidly staining, central body.

FIG. 10.—Form containing a ring-shaped central body.

FIGS. 11, 12, 13, 14, and 15.—Apparent division forms of 10.

FIG. 16.—Small body containing central body and chromatoid bodies.

FIGS. 17 to 33, inclusive.—Larger "Negri body" and possible division forms from it.

FIGS. 34, 35, and 36.—Bodies containing larger central body and smaller chromatoid granules.

FIGS. 37 to 55, inclusive.—Still larger forms with their possible divisions. The frequent ring-shaped arrangement of the chromatin in the central body and the karyosome-like structure within it are more apparent.

FIGS. 38 to 42, inclusive.—Apparent fragmentation of the chromatin and the formation of buds.

FIGS. 54 and 55.—Show similar budding from different forms. In Fig. 55 the elongation of the chromatin granules is marked.

FIG. 56.—Form apparently made up of many tiny indefinite bodies.

PLATE 18.

Fig. 1.



Fig. 2.

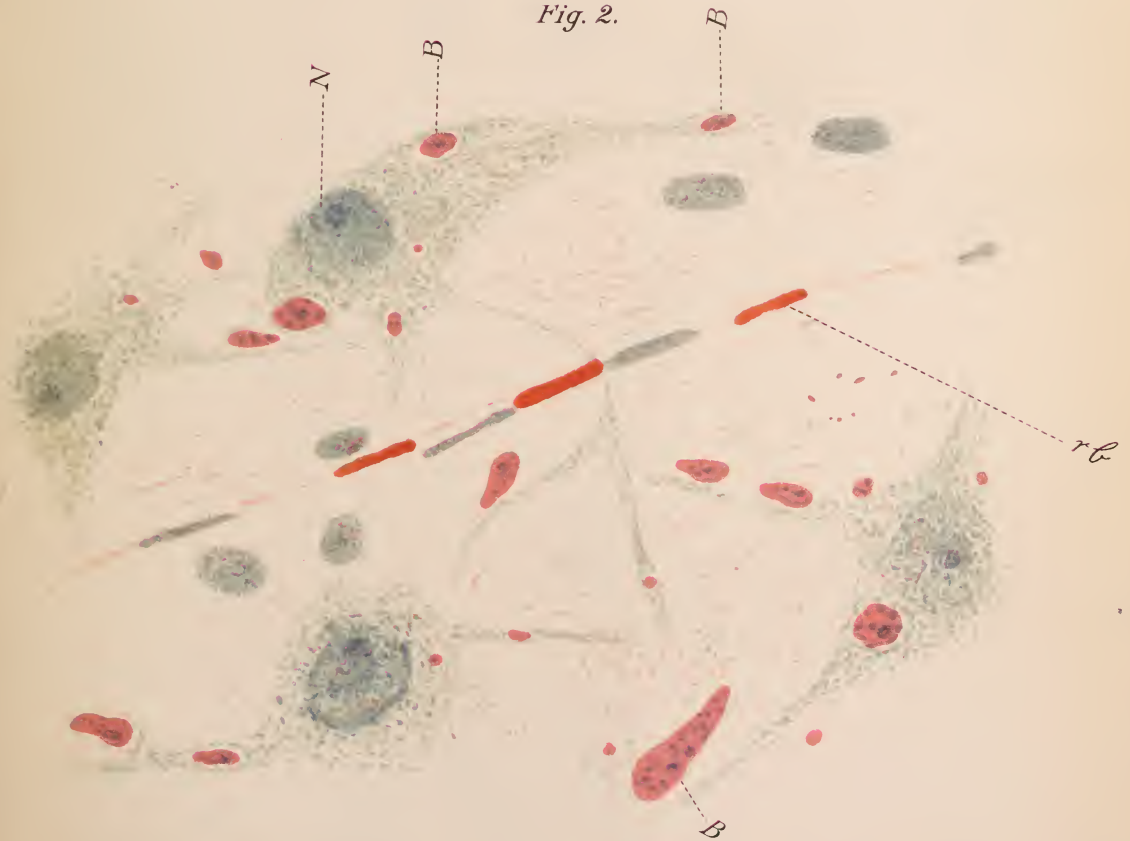


PLATE 19.



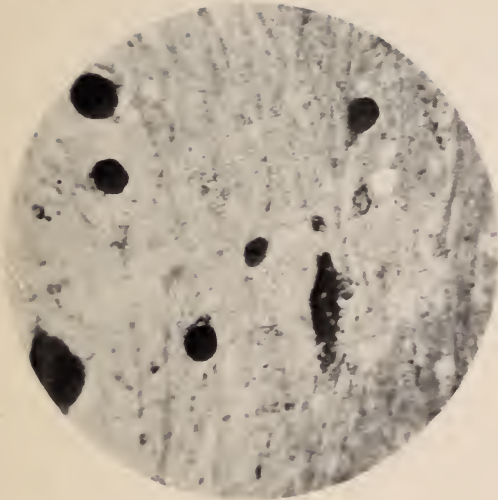


FIG. 1.

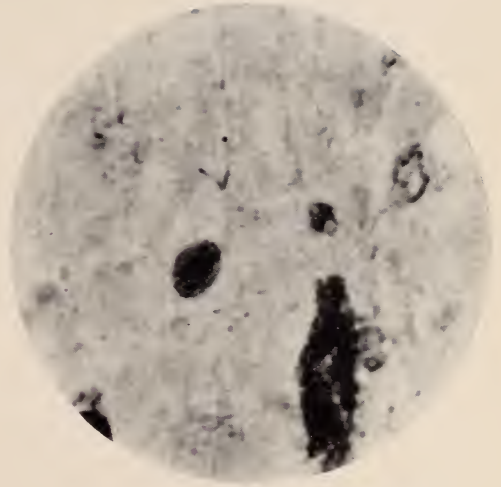


FIG. 2.

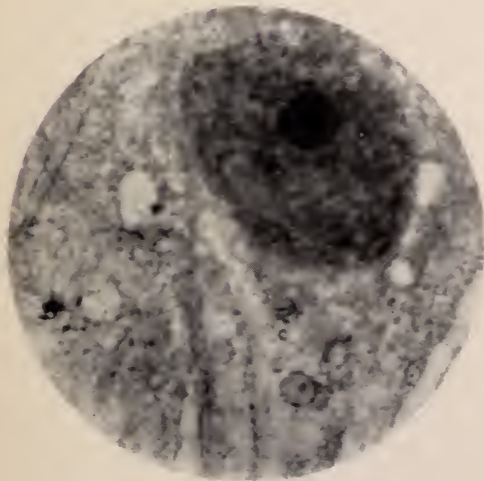


FIG. 3.

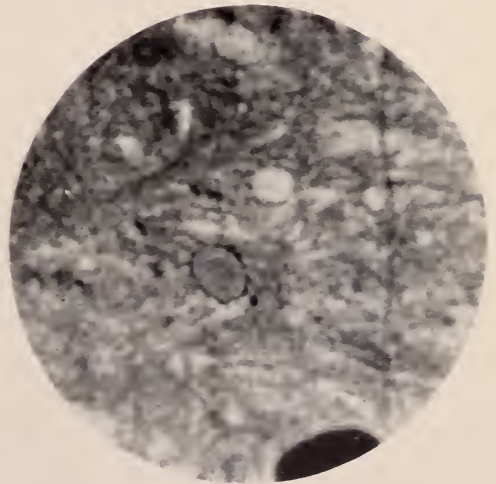


FIG. 4.

PLATE 21.

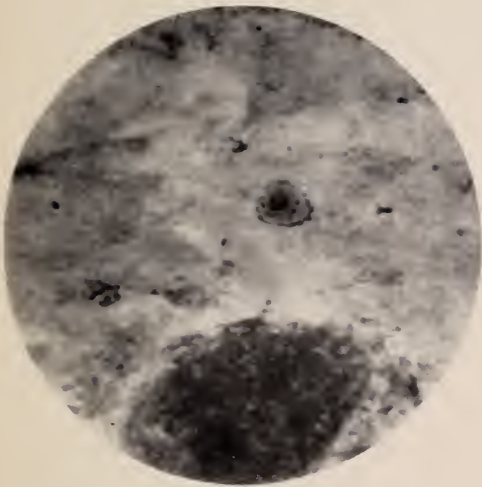


FIG. 5.

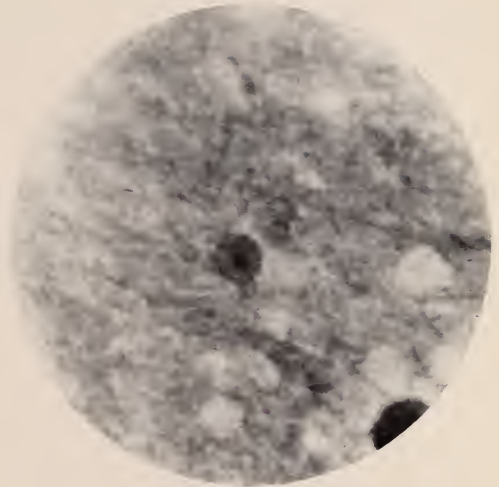


FIG. 6.

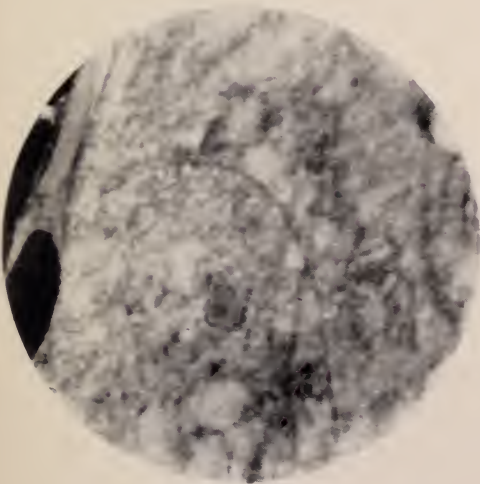


FIG. 7.

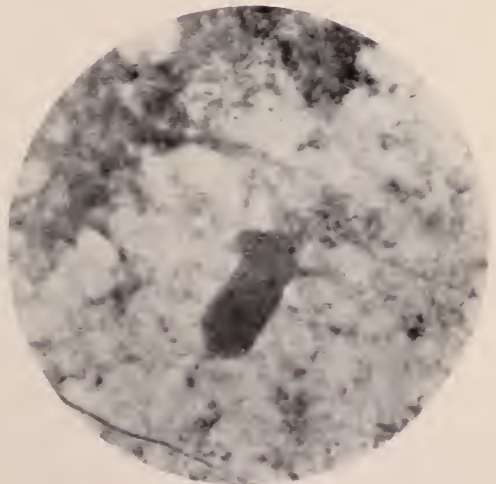


FIG. 8.

DESCRIPTION OF PLATES 20 AND 21.

All the photographs were taken from smears stained with Giemsa's solution.

FIG. 1.—From Ammon's horn, street rabies, oval "Negri body" showing central body and chromatoid granules. $\times 1,200$.

FIG. 2.—The same body. $\times 2,000$.

FIG. 3.—Two small structured "Negri bodies" in cytoplasm near nucleus of nerve cell. One slightly out of focus. From Ammon's horn, street rabies. $\times 2,000$.

FIG. 4.—An oval "Negri body" pressed out from branch of cell shown in Fig. 3. The "body" contains large oval central body about which is ring of small elongated chromatoid granules. $\times 2,000$.

FIG. 5.—A rounded "Negri body," showing well the complete circle of chromatoid granules about the central body. From street rabies. $\times 2,000$.

FIG. 6.—Another rounded form from the same case.

FIG. 7.—A triangular form showing central body and irregular chromatoid granules, from same case.

FIG. 8.—Budding form, from Ammon's horn of dog which died 20 days after subdural inoculation of human rabies.

The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 3

June 30, 1906

No. 4

A STATISTICAL STUDY OF GENERIC CHARACTERS IN THE COCCACEÆ.*

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ASSISTED BY

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ANNIE P. HALE.

- I. PURPOSE OF THE INVESTIGATION.
- II. METHODS OF THE INVESTIGATION.
 1. Isolation of Cultures.
 2. Selection of Characters for Study.
 3. Morphological Characters.
 4. Cultural Characteristics.
 5. Biochemical Reactions.
- III. RESULTS OF THE INVESTIGATION.
 1. Habitat.
 2. Grouping of Cells, and Dimensions.
 3. Gram Stain.
 4. Surface Growth.
 5. Fermentation of Carbohydrates.
 6. Reduction of Nitrates.
 7. Optimum Temperature.
 8. Chromogenesis.
 9. Gelatin Liquefaction.
- IV. CONCLUSIONS FROM THE INVESTIGATION.
 1. Foundation of Subfamilies and Genera among the Cocci.
 2. Systematic Summary.
- V. REFERENCES.

I. PURPOSE OF THE INVESTIGATION.

There has been placed in the hands of the biologist within the last few years a new instrument of research of the highest value. This is the statistical method, first suggested for the study of human characteristics by Quetelet (1846), specifically applied to the bio-

* Received for publication April 3, 1906.

logical problems of variation and heredity by Galton (1889), and extended and developed in detail by Pearson and his pupils. The most important papers on this subject may be found in the files of the *Philosophical Transactions of the Royal Society of London* and in *Biometrika*. Admirable brief summaries have been prepared by Pearson (1900) and Bigelow (1904).

In many fields of science the statistical method, in its strict sense, is not applicable. Where laboratory experiments may be made, as in most fields of physics and chemistry, a comparatively small array of data obtained under perfectly controlled conditions may permit the derivation of laws of relationship without extensive statistical analysis. The same thing is true in certain fields of biological research. As soon, however, as we proceed to the subtler problems of evolution, it becomes necessary to accumulate a large number of observations and to analyze them by recognized statistical methods. These methods alone have brought order out of chaos in anthropology (Ripley, 1899). They have laid the first foundation for a real science of mental and social phenomena (Thorn-dike, 1904; Woods, 1906). They offer the most promising clue for tracing the true relationships among the lower forms of plant and animal life.

As we have elsewhere pointed out, the classification of the bacteria presents peculiar difficulties.

Morphological distinctions are so slight that physiological characters must necessarily be invoked in order to separate and classify the various organisms, and these physiological characters are often variable. Pathogenicity may be taken as a type of those powers of the organism which are easily and profoundly modified by external conditions. On the other hand, there are numerous characters which appear to be extremely constant. Such minute differences as occur in the resistance of different races to unfavorable conditions often remain unchanged through long periods of cultivation. In using these constant characters for classification we are met by another difficulty. Though constant, the differences are very minute, and in studying a number of organisms a perfect gradation is often found between the widest extremes. This is exactly what should be expected from organisms which reproduce only by asexual methods, since it is the fusion of independent cells which swamps minor differences producing the uniformity of species among higher plants. With asexual reproduction every minute variation which is inheritable must persist unchanged until some other chance variation occurs. Each such variation means a new and different type of bacterium.

The immense number of generations which may succeed each other in a short space of time makes boundary lines as shifting as they would become among the higher plants if a dozen geological epochs were considered all at once.

Since with unicellular organisms acquired characters may probably be inherited in a higher degree than with other forms, existing races of bacteria will be markedly

influenced by the selective effect of environmental conditions, and must bear the impress of their recent history.

There are, therefore, no species among the bacteria in quite the sense in which we ordinarily use the word—as indicating a group of individuals bound together by a number of constant characters and easily identified by mutual fertility. From one point of view each distinct race might be considered a species; but to apply a name for every grade of difference in each varying character would be impracticable; and such names could have no true specific value. The best solution of the difficulty is the establishment of certain types around which the original organisms may be more or less closely grouped; but it must be clearly recognized that the groups thus formed are defined by relation to the type at their center and are not sharply marked off at their extremities from the other groups adjacent.¹

For these reasons the science of systematic bacteriology has remained in a notably undeveloped state. A score of large groups of bacteria have been more or less satisfactorily recognized by Flüggé (1896) and others. Certain of these groups, like the *aërobic* spore-formers, the colon bacilli, and the diphtheria bacilli, doubtless represent true natural families or genera. In one such group, that of the *aërobic* spore-formers, where appreciable morphological differences exist, the species and varieties have been carefully worked out by Chester (1904). Far too many specific names among the bacteria however, mean less than nothing. The incomplete description of a vast number of identical or minutely differing forms has led to a confusion quite disheartening to the student of such systematic works as those of Migula (1900) and Chester (1901). Among the Coccaceæ we have compared the published descriptions of 445 species and found evidence for only 31 distinct types (Winslow and Rogers, 1905). These are defined mainly by arbitrary combinations of the three characters of acid production, chromogenesis, and the liquefaction of gelatin. It is small wonder that most bacteriologists have abandoned any attempt at a natural classification, and have sought refuge in such frankly arbitrary schematic groupings as those of Fuller and Johnson (1899), Weston and Kendall (1902), and Jordan (1903). The same tendency carried to its extreme is shown in the decimal systems of Gage and Phelps (1903), and Kendall (1903), and in the modifications recently adopted by the Society of American Bacteriologists.

These systems are most valuable for a routine descriptive work, and for arranging and cataloguing records of cultures. They may, however, lead to error, unless used with due caution. In the first

¹ WINSLOW AND ROGERS, 1905

place, the determinations on which such schemes are based are usually qualitative only and not quantitative. In the second place, the application to all bacteria of one fixed series of characters arranged in an arbitrary order tends to suggest a mechanical view of bacterial relationships which is very far from the complex truth.

In order to obtain a just idea of the real relations of organisms, it is necessary to consider each systematic group by itself. As Robinson has pointed out in an admirable paper on generic classification (Robinson, 1906), "a difference having great classificatory significance in one place may be almost valueless in another." In studying any one group it is therefore necessary to examine afresh each of the various characters used for the identification of bacteria in general, and to determine its local value and significance. Secondly, under each character it is necessary to determine how many distinct types of structure or function may occur. This can be done only by measuring the character quantitatively in a large series of individuals, and plotting curves of frequency which will show whether the individual forms fluctuate about one or several modes. This has been attempted by Howe (1904) with good results, for the composition of the gas produced in dextrose broth by organisms of the *B. coli* group.

Finally, the correlation between various properties should be determined, since it is obvious that the presence of several distinct characters in association is generally of more significance in classification than that of any one alone.

In the present study we have attempted to obtain the data indicated, for certain groups of the Coccaceæ. We have measured the easily and definitely measurable, variable characters in 500 separately isolated races of organisms, and analyzed the data obtained, with two ends in view. We have first plotted the frequency curve for each character to find whether the array varies about one or several modes, and where the modes are situated, with some measure of the extent of variation about these centers. In the second place, we have calculated correlation factors for the most significant pairs of characters. Each mode on the curves of frequency may fairly be taken to mark a natural species or variety, and the characters which vary together must form the most important basis for the establishment of the larger groups. By such a method alone it is

possible to locate those mountain peaks in the chain of bacterial variations which rightly deserve generic and specific names, although records of the characters of individual races by the decimal system are of the greatest value in mapping out intermediate regions. Only the statistical study of numerous individuals by comparable quantitative methods can reveal the general laws of natural classification among the bacteria; and this study must be made in each group with an open mind free from arbitrary predispositions.

We desire in advance to deprecate a comparison between the present work and the numerous detailed and exact biometrical studies which have appeared in other fields. In bacteriology our methods of measurement are crude and tedious, and the general knowledge requisite for the selection of a homogeneous mass of material is lacking. We should know the outlines of the general groups of the cocci, for example, before we can properly select material to study variation in any one of them.

II. METHODS OF THE INVESTIGATION.

I. ISOLATION OF CULTURES.

With regard to the larger groups of the Coccaceæ we have elsewhere shown (Winslow and Rogers, 1905) that the family could be divided into two subfamilies and five genera, defined as follows:

Subfamily 1, Paracoccaceæ (Winslow and Rogers): Parasites (thriving only, or best, on, or in, the animal body). Thrive well under anaërobic conditions. Many forms fail to grow on artificial media; none produce abundant surface growths. Planes of fission generally parallel, producing pairs, or short or long chains.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule.

Genus 2, *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large groups or packets). On agar streak effused, translucent growth, often with isolated colonies. In stab culture little surface growth. Sugars fermented with formation of acid.

Subfamily 2, Metacoccaceæ (Winslow and Rogers): Facultative parasites or saprophytes. Thrive best under aërobic conditions.

Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cells aggregated in groups, packets, or zoöglea masses.

Genus 3, *Micrococcus* (Hallier) Cohn: Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Acid production variable.

Genus 4, *Sarcina* (Goodsir): Saprophytes or facultative parasites. Division under favorable conditions in three places, producing regular packets. Sugars as a rule not fermented.

Genus 5, *Ascococcus* (Cohn): Generally saprophytic and cells imbedded in large, irregularly lobed masses of zoöglea, in process of carbohydrates. Acid usually formed.

In the present investigation we have included representatives of only three of these genera. The organisms belonging to the genus *Diplococcus* do not lend themselves to comparative study on account of the difficulty with which they may be cultivated, and representatives of the genus *Ascococcus* occur, if at all, only in certain peculiar habitats. We have limited our study to forms which can be found in ordinary environments, and which may be cultivated on ordinary laboratory media; that is, to the genera *Streptococcus*, *Micrococcus*, and *Sarcina*.

We have procured our cultures in approximately equal proportions from five different sources: from the internal tissues of the diseased human body, from the outer surfaces of the normal human body, from water, from earth, and from air. Cultures classed under Habitat I, the tissues of the diseased body, were obtained chiefly from the Boston City Hospital, and the Massachusetts General Hospital, of Boston, and the Johns Hopkins Hospital, of Baltimore. We desire to express our cordial thanks to the bacteriologists of these institutions for their courtesy in furnishing us with these organisms. The cultures classed under Habitat II, surfaces of the normal body, were obtained from three sources. A considerable number were isolated from serum tubes, received by the Boston Board of Health for diphtheria diagnosis. In this connection we desire to acknowledge the courtesy of the bacteriologists of the Board. Only those cultures which gave a negative diagnosis for diphtheria were used. Another series of cocci was isolated from the hands of students

in the Massachusetts Institute of Technology. In collecting them each subject rubbed the front and back of one hand with a wet wad of sterile cotton, running the wash water into a sterile cup. Finally a small number of cultures were obtained from excreta of man and animals. Under Habitat III cultures were obtained from a wide variety of natural waters—public supplies, streams, ponds, pools, shallow wells, driven wells, and the sea. Samples were taken as far as possible only from sources held to be free from pollution. Under Habitat IV organisms were isolated from various samples of earth, loam, clay, sand, etc., obtained mainly in different regions of eastern Massachusetts. The cultures grouped under Habitat V were taken from plates exposed to the air, indoor and out, and here are also included certain organisms of unknown origin which appeared as contaminations, or for whose previous history we have no record.

In each case the sample to be studied was first plated on agar and incubated at 20°. Colonies which looked like cocci (not possessing, that is, the characters of such well-marked forms as *B. mesentericus*, *B. Zopfii*, or *B. fluorescens*) were fished to agar streaks; from each sample only one culture was taken, unless several distinct types of colonies appeared. The agar streak cultures were examined under the microscope and, if apparently cocci, were replated in order to insure their purity, again transferred to agar streaks, and again examined under the microscope. All this preliminary work was carried out at 20°, and the stock cultures finally obtained were kept on agar at the same temperature. There can be no doubt that by this method of procedure we failed to obtain many of the more strictly parasitic streptococci which grew only feebly on solid media and are most active at a temperature of 37°. This fact must be taken into account in interpreting our results. For *Micrococcus* and *Sarcina*, however, the series should be fairly representative.

2. SELECTION OF CHARACTERS FOR STUDY.

The characters ordinarily used in descriptive bacteriology are few, particularly in a group of such simple morphology and limited biochemical powers as the Coccaceæ. This number must be still further reduced, however, when we come to inquire which of them really indicate constant and independent variations. In the first place, it

is necessary to eliminate properites which are due mainly to the character of the medium and the conditions of incubation. As we shall show later, those minute differences in the appearance of colonies on gelatin which form the basis for a large number of German descriptions, fall mainly under this head. Secondly, many characters, while really belonging to the organism itself at a given moment, are so easily modified by cultivation under other conditions as to be practically worthless in systematic work. Among the cocci, pathogenicity is a property of this sort. In the third place, it is evidently unfair to give independent weight to characters which are simply the indirect result of other properties already recorded. Thus among the cocci differences in broth cultures are closely connected with the size of the cell aggregates. Organisms growing in large groups, like most of the sarcinæ, produce heavy sediment and often colony-like groups on the walls of the tube, while those in which the cells readily separate exhibit a more diffuse turbidity. Plate cultures add little more information than may be obtained by a careful scrutiny of stabs and streaks; and the growth on potato and blood serum in many groups of bacteria, and particularly among the cocci, are only valuable as measures of that extremely fugitive quality, the general vigor of the culture.

The considerations which have influenced us in the selection of characters for study among the Coccaceæ may be conveniently arranged in the order, and under the headings, of the Report of the Committee on Standard Methods of Water Analysis to the Laboratory Section of the American Public Health Association (1905).

3. MORPHOLOGICAL CHARACTERS.

Form.—The form of the individual cell furnishes no help in the classification of the Coccaceæ, since under favorable conditions all appear as regular spheres. Irregular oval forms occur at times, particularly in cultures freshly isolated from the throat or alimentary tract, but the form usually becomes normal after cultivation.

Manner of grouping.—The grouping of the cell elements offers a character of considerable importance among these bacteria. While the cocci do not exhibit an entirely unchanging form of grouping, the individuals do show a distinct tendency to occur in one of four forms—either in pairs, chains, masses, or packets.

The grouping is somewhat influenced by the age of the culture and by the kind of medium on which it has grown. Even the same culture will show wide variation from the typical arrangement of the elements. For instance, streptococci occur singly, in pairs, chains, and small masses; but the most frequent arrangement, and that obtained under the most favorable conditions (in liquid media), is in chains. Again, sarcinæ occur singly, in pairs, and in small masses as well as in packets, yet the typical form is the sarcina-pocket. Cocci grown on Nährstoff regularly occur in plates, and usually capsulated ones.

In a number of preliminary studies we compared the groupings of the same cultures in various media and under various conditions, examining cultures of different ages, from nutrient broth, sugar broth, peptone solution, hay infusions, nutrient agar, and gelatin, and acid and alkaline gelatin. Cultures more than two weeks old showed abnormalities both in the individual cell and in its groupings. With this exception, the differences produced were very slight. The only constant effect of the medium upon grouping which was apparent was a more distinct development of chains in liquid cultures. Organisms which appear as long chains in fresh broth cultures may show only short chains with irregular groups on solid media. In the present study we have omitted the broth morphology for lack of time, and have recorded the grouping only as apparent on the agar streak.

The streaks used were never more than three days old, and the grouping was observed after staining lightly with methylene blue and mounting in cedar oil. Too heavy staining may introduce a serious error by making packets of small sarcinæ appear like large single cells. These observations on the culture stained with methylene blue were controlled by careful observations of the slides prepared for the study of the Gram stain, as noted later.

We have distinguished two main groupings only by this method of examination. The occurrence of packets marks one, and the absence of packets the other, group. In the first group occur the streptococci, which produce pairs, long chains, and irregular groups; and the micrococci, which show pairs, short chains, fours, and irregular groups; while the sarcinæ include organisms which produce fours, irregular groups, and packets, as well as those

extreme forms which show only packets. None of these differences but that between the presence and absence of packets appear on agar with sufficient constancy to be determined definitely. For distinction between streptococci and micrococci the observation of broth cultures would perhaps be valuable.

Dimensions.—The cocci exhibit a range in size from 0.1 to 2.0 μ with considerable variation between individual cells in the same culture. We were somewhat surprised to find that we could demonstrate no definite relation between size and the age of cultures, or the conditions of cultivation. In a series of preliminary studies the same organism was grown on seven kinds of media and examined at intervals during a period of two months. The maxi-

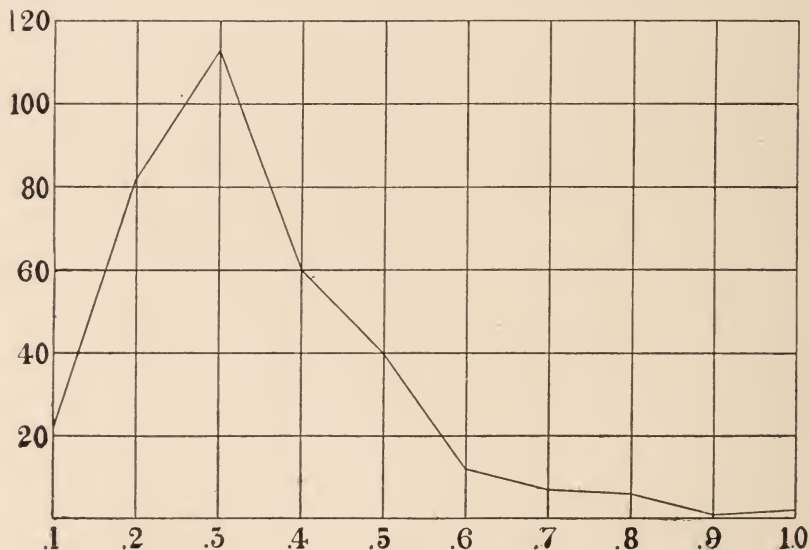


FIG. 1.—Dimensions of 345 cocci. Abscissæ, average diameter in μ . Ordinates, number of cultures.

mum size, in different cultures, was recorded on the first, second, seventh, 14th, 42d days, and after two months respectively. The maximum size developing in the different kinds of media during those two months was found, respectively, in broth at 37°, broth at 20°, Nährstoff-Heyden, nutrient gelatin, acid and alkaline gelatin, and under anaërobic conditions. In other words, the age and kind of medium had no constant effect, except that in most cases the Nährstoff and other poor media showed the smallest individuals. No

constant difference in size was apparent in comparing solid and liquid cultures. One series of organisms examined in dextrose broth and on agar, at periods ranging from one day to two weeks, showed the same average size in both media and at all ages. Finally we attempted to see whether prolonged cultivation under special conditions would affect the size of the cell. Cultures were grown for 10 days, in broth at 37°, on nutrient gelatin, and on acid and anaërobic gelatin with daily transfers. The size of each culture was recorded on the 10th day, after which time each was transferred to gelatin and examined after one day. The results showed practically no significant differences.

In a comparison of the size as determined by examination of living organisms and of stained preparations, the cells appeared generally somewhat smaller after staining. This is no doubt partly due to some shrinkage in drying, and partly to the imperfect definition which makes the unstained specimens appear larger than they really are. Occasionally, when the staining was too heavy, the stained cells appeared larger. In any case the differences are unimportant, and we have used the size of the methylene-blue-stained preparation throughout our work.

Staining reactions.—Since the cocci, as far as we have examined them, all stain easily with methylene blue, we have made no special tests with anilin-gentian-violet. The Gram stain has, however, been used on all our cultures, since, in the genus *Diplococcus* and in many other groups, it has been thought to have such special importance.

The value of this staining method has been studied with considerable care by Mr. A. T. Brant, working in the laboratories of the Institute. Mr. Brant found, as other observers have done, that while certain bacteria are constantly Gram-negative or Gram-positive, others exhibit an intermediate condition, retaining the stain under some conditions and giving it up under others. In his, as yet unpublished, paper he notes, for example, that all cultures of *B. coli* are decolorized by one minute's treatment with alcohol, while *B. megatherium* constantly fails to decolorize after three hours. On the other hand, with *B. fluorescens*, *M. pyogenes*, *M. aureus*, and *B. diphtheriae* the result is affected by the time of decolorization, as well as by the age of the cultures. Between the fixed points at the

extreme, preparations will yield varying results, showing some cells stained and others decolorized. As a rule, the large majority of cells in a given preparation will show one reaction or the other; but a second slide made from a similar doubtful case might yield a different result.

The time chosen for decolorization is, of course, an arbitrary factor which will affect the proportion of positive results obtained. In our work, as a result of Mr. Brant's experiments, we fixed on three minutes, although we are not certain that this is really preferable to the five-minute period fixed by the Committee on Standard Methods. We have applied the anilin-oil-gentian-violet for one and a half minutes, and the Gram solution for one and a half minutes instead of the one- and two-minute periods of the committee.

In all cases we made the stain on young 20° agar cultures (not over five days old), and in each case the test was made in duplicate at different times. When the results of the two tests coincided, the culture was recorded as positive or negative. Cultures which gave one positive and one negative test, or in which the stained and decolorized appeared in about equal proportions, are recorded in an intermediate class.

Flagella.—As a result of the work of Ellis (1902), we have devoted considerable time to the study of motility among the cocci. This author reported the finding of spores and flagella in various streptococci and sarcinæ, and Arthur Meyer carried this position to an extreme in the statement that "the researches of Ellis have rendered it doubtful whether there are any species of bacteria which entirely lack flagella" (Meyer, 1903). We examined a number of cultures very carefully, transferring them at frequent intervals on different media, according to the general plan adopted by Ellis. We found in almost every case active vibratory movements, with a tendency to incomplete rotation, the successive jerks sometimes producing a gradual translation across the field. This type of behavior is entirely different from the true motility characterized by slow, steady revolution, which appears in such forms as *S. agilis*. We are convinced that most of the cocci are non-motile, while a few forms show true movement; it is with this type of motility that clearly stainable flagella have been found associated. The study of this character is

therefore of significance. It is questionable, however, whether it is one of the most important characters in this group of bacteria. It appears from the published descriptions of species that this property is not correlated with any other character, arising independently in forms exactly resembling non-motile forms in every other respect. On account of its rarity and this apparent lack of correlation with other differences, as well as on account of the difficulty of studying it, the property of motility has been so far omitted from the present study.

Spores.—The experiments carried out by Ellis (1902) strongly suggest the presence of specially resistant cells in old cultures of the cocci. His figures are, however, by no means conclusive as to the existence of true spores. In the absence of any observations as to germination, we have not felt that the evidence warranted extensive microscopic study of this character.

Fission.—A study of the conditions influencing the growth-forms of the Coccaceæ should be of considerable interest. Pairs and chains are apparently associated with meager, and groups and packets with more abundant, development. The effect of the general rate of growth must, however, be modified by the rate at which cell-wall and cell-protoplasm, respectively, are formed.

A careful study of the method by which these groupings arise in cell-division, such as could be made by the use of Hill's hanging-block method, would no doubt throw much light on all such points, and should precede any final conclusions as to the relationships of the cocci. In examining a large number of organisms, however, the agar block would have proved too time-consuming. We have therefore limited ourselves to the observations made on stained preparations from ordinary cultures.

Capsules.—Considerable preliminary work failed to indicate any constant differences in capsule formation among the cocci studied. This character appears to be of considerable value among the diplococci (Buerger, 1904); but even with them it varies markedly with the medium used for cultivation. We cultivated certain selected organisms in broth at 20° and at 37°, on nutrient gelatin, acid gelatin, alkaline gelatin, anaërobic gelatin, and Nährstoff-Heyden agar, and examined them at intervals by Welch's staining method. In every case capsules were apparent at some stages, being most

strongly developed in old cultures and on poor media like the Nährstoff agar. This character has not seemed to us of sufficient diagnostic value to be included in our routine examinations.

Involution and degeneration forms.—In numerous examinations of old cultures we found no involution forms of special significance. As noted above, swollen and oval forms are more apt to occur in old cultures of cocci, but they are not sufficiently definite to warrant record.

4. CULTURAL CHARACTERS.

In a study of this sort we have necessarily included only those tests which reveal definite and independent variable characters. Most of the commonly observed cultural characteristics are the secondary results of a few fundamental properties which can be observed on one medium as well as on several. For this reason we have eliminated a number of the ordinary media from our routine. The general character of the growth is approximately the same on *agar*, *blood serum*, *potato* or *Nährstoff*, except that agar has always markedly more growth and potato often none. An organism producing abundant chromogenic growth on agar will give good growth and some pigment on the other media. The streptococcus growth on agar gives restricted and veil-like growth on serum and Nährstoff, and usually no growth on potato. In other words, Nährstoff agar, serum, and potato are simply poorer media than agar, and show no specific characteristics other than those due to feebleness of growth. Blood serum may be useful in other groups to show a special type of liquefaction, but in a preliminary study of 50 of our cultures we never found this to occur, and it is nowhere recorded in published descriptions of the Coccaceæ. In 25 out of 50 cultures grown on potato no growth occurred, and in no case have we observed discoloration. These media have therefore been omitted. This action is in accordance with the conclusions of the Committee on Standard Methods (1905), in considering their value for general diagnostic use.

Nutrient broth.—In the group of the cocci we have not found that any information of definite value could be derived from a study of broth cultures. None of the forms studied form a surface pellicle or produce any characteristic odor. There remain to be observed only two features—turbidity and sediment—which in our

judgment depend directly on other properties, such as the general vigor of growth and the size of the cell aggregates. Both turbidity and sediment vary markedly with the age of the culture; what is first turbidity later settles to form sediment, as the waste products of the bacteria check their development. The amount of either depends on the activity of growth. A constant difference often appears between cultures which early in the course of development show considerable turbidity with little or no sediment, and those which almost at once develop a heavy sediment with colony-like masses

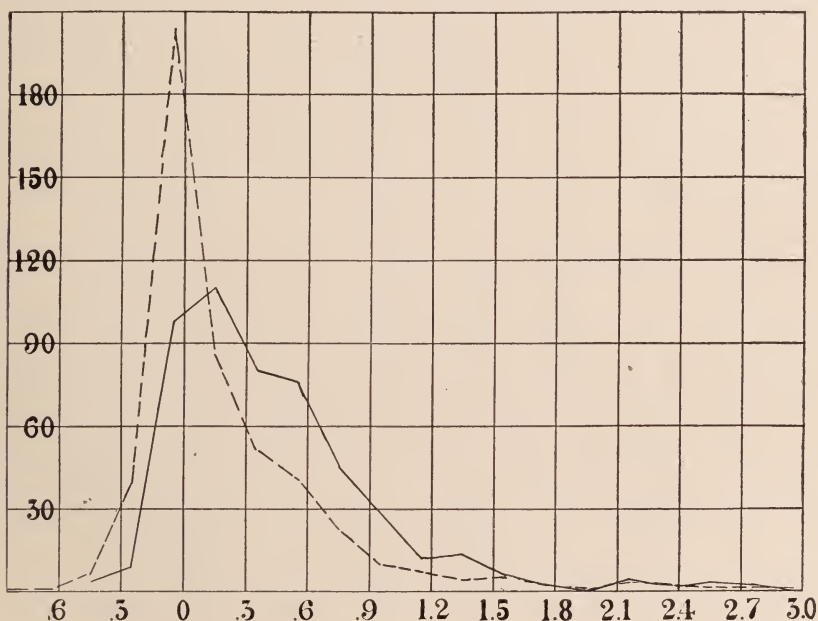


FIG. 2.—Acid production of 500 cocci; in dextrose broth = —————, and lactose broth = - - - - -. Abscissæ, acidity in per cent normal. Ordinates, number of cultures.

of growth clinging to the walls of the tube. This difference, however, appears to be correlated with the growth-form and general vigor of the coccus. Organisms of the *Streptococcus* type with cells separating readily, which show faint surface growth, produce chiefly turbidity; while organisms like *Sarcina* with large cell aggregates and rich surface growths, show heavy sediment.

Gelatin plates.—Minute differences in the macroscopic and microscopic appearance of colonies on gelatin are given great

weight in German systems of classification. Certain special characteristics do, indeed, appear in old gelatin colonies of the cocci after several weeks of incubation. Colonies may remain almost spherical; or they may expand in flat, disclike growths with terraced edges. Sometimes a distinct boss appears at the center, surrounded by a flatter area. The edges may be entire, or more or less deeply scalloped, and the edges of the scallops may be produced inward in folds. Concentric rings sometimes appear in the interior of the colony, or zones of partially liquefied gelatin around its periphery. Some of these characters vary without any apparent reason, as different colonies on a plate show different characteristics; this is perhaps due to differences in the position of the original cell relative to the gelatin surface. Most of them are profoundly modified by variations in the amount of moisture in the gelatin and in the atmosphere above. In a series of comparative studies with different conditions of incubation we found that highly characteristic colonies of granular structure, with deeply lobed edges and indented surfaces, could be produced by cultivation in an incubator whose atmosphere was kept dry by calcium chloride. Dunham (1903) has pointed out the wide differences which may be due to slight variations in the physical properties of the gelatin used. Those differences which are really characteristic of the organisms themselves appear to be related to two fundamental powers: the general vigor of growth and the liquefying power. It may be possible that other differences exist in old gelatin colonies which are really characteristic, but in the present state of knowledge it seemed best to omit the gelatin plate in favor of more definite tests. Liquefying power and general vigor of growth are observed in the gelatin stab and the agar streak respectively.

Gelatin tubes.—All our cultures have been studied in the gelatin tube, but only the single character of the amount of liquefaction has been systematically recorded. The distinction between different non-liquefying colonies lies in the amount of surface growth and the color, both of which characters are more easily studied on the agar streak. The character of the surface growth, like that of the gelatin plate colony, does not appear in this group to offer any character of diagnostic value, and all the cocci grow fairly well in the stab.

Among the liquefying forms we have not found the shape of the liquefaction of sufficient constancy to be recorded. Whipple (1902) has strikingly shown the uncertainty of this character—almost every possible type appearing in media made with slightly different commercial gelatins. The Committee on Standard Methods (1905) has also omitted this property.

The amount of liquefaction of gelatin was therefore the only character recorded on the gelatin stab. The method by which this was measured will be described under "Biochemical Reactions."

Agar plates.—The same reasons which led us to omit the gelatin plate militate against the use of the agar plate as a diagnostic test. Constant differences which exist between colonies are slight and depend on a few fundamental properties which may be more easily observed on other media, notably on the agar streak.

Agar tubes.—The general conclusion from what has been said in this discussion of cultural characteristics is that in the cocci a single medium is sufficient for their determination. We should, however, deprecate any extension of this conclusion to other groups where the gelatin stab or the plate culture may yield information of definite value. Even among the cocci further study may show constant and characteristic differences in gelatin colonies, and if this should be the case, no one could fail to welcome an addition to the meager list of diagnostic characters at our disposal. In the absence of evidence as to the value of these media, we feel it unwise to repeat tests mechanically and without any definite purpose, merely because they have had an important place in the historical development of the science.

All cultural characteristics have therefore been observed in the agar tube. A combined streak and stab was made on a slant surface, and the cultures were uniformly studied after incubation for two weeks at 20° C. Cultures of different age exhibit marked differences, but the characters of the old cultures are the outcome of those of the new. Comparative studies with lactose agar and glycerin agar showed neither to be as favorable a medium as ordinary nutrient agar.

In order to obtain a comparative idea of cultural characters we examined two weeks' agar streaks of the whole 500 cultures

at the same time. We are somewhat surprised to find that the visible differences between the cultures were due almost wholly to two properties—chromogenesis and the general vigor of surface growth. There was a distinction in luster between a large majority of the

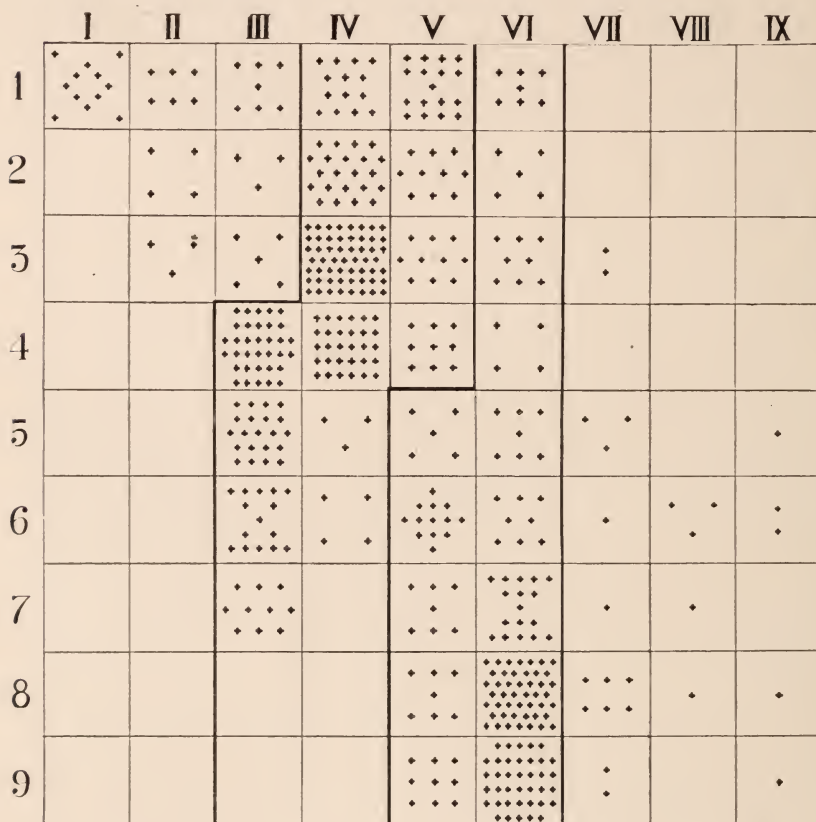


FIG. 3.—Distribution of 500 cocci according to chromogenesis. Roman numerals, hues; Arabic numerals, chromos.

cultures which had smooth and shining surfaces, and a few which were dull and rough. This difference appears, however, to be due simply to the relative amount of growth and moisture in the tube. Faint growths are moist and shining, while heavy growths in tubes which do not contain much moisture show the dry, rough, dull appearance. The “white chromogens” showed another slight difference, varying from an opaque porcelain white to a duller and

more translucent growth of indefinite color and somewhat shiny appearance; but there was no sharp boundary to separate the types. For the present we have omitted this character, although it may prove to be of importance in more detailed work.

We have therefore noted, as cultural characters on the agar streak, only the color production and the vigor of surface growth. The method of studying the former character will be described under "Chromogenesis." Under "Vigor of Surface Growth" we found it possible to distinguish five different types. Grade 1 includes forms of the *Streptococcus* type which form only a very faint, veil-like, growth, or a few translucent dotted colonies on the surface. Grade 2 is reserved for a somewhat more abundant, but still meager, growth. Grade 3 corresponds to a good, but not abundant, streak; Grade 4, to an abundant growth; and Grade 5, to a very heavy surface development.

The relation to free oxygen is distinctly involved in the vigor of surface growth, and the agar streak also served for the study of various other biochemical reactions. Inhibition of growth by acidity and alkalinity of media, temperature relations, and pigment formation were all recorded on this medium under conditions to be described below.

5. BIOCHEMICAL REACTIONS.

Action upon milk.—Milk is a favorable nutrient medium for bacterial growth because of its rich food properties, and in many groups it gives important information, but it has no specific diagnostic value for the Coccaceæ, as all the changes it undergoes are correlated with those which occur in sugar broths and with the general activity of the organism. No coagulating enzymes and casein-digesting enzymes are found in this group, so far as we are aware, and no gas or odor is produced. The only changes which the cocci effect in milk are therefore the production of acid or alkali, coagulation and decolorization of the litmus.

Decolorization has no significance, except that it indicates the general activity of the organism. When the organism is most active, it uses up the oxygen and reduces the litmus, which is accordingly decolorized, and, conversely, when activity grows less, oxygen diffuses from the surface making the litmus pink again.

Coagulation depends upon the amount of acid produced, and is more easily studied in sugar-broth cultures.

Action upon carbohydrates.—The characteristics usually observed in sugar broth are turbidity and sediment, relation to oxygen, gas production, and acid production. We have given reasons, in discussing nutrient broth, for considering turbidity and sediment unimportant, and the relation to oxygen is most sharply defined by surface growth in the agar tube. None of the cocci, so far as known, produce gas, and there remains only acid production to be recorded. For this purpose ordinary straight tubes were used. The sugars tested were dextrose and lactose. Saccharose has been omitted for the present, for lack of time. A preliminary test indicated that this sugar is less commonly fermented by the cocci than are dextrose and lactose.

The media were made up in the usual manner with 2 per cent of the sugar to be tested. The reaction was made about neutral, and after tubing and sterilization it was usually between 0.5 and 1.0 per cent. After standing for two weeks sterile blanks showed a slight further rise in acidity, so control tubes were always kept with each batch inoculated and titrated at the end of the experiment. After considerable preliminary experimentation, it was decided to titrate with phenolphthalein as an indicator in the cold. Methyl orange is not sensitive to the organic acids and gives a poor end-point. With phenolphthalein a comparative series of titrations made on the same tubes, first cold and then boiling, showed slightly higher results by the latter method. Evidently heating increases the apparent acidity more by the breaking-up of unstable compounds than it decreases it by driving off carbon dioxide. The cold method was therefore used. To 5 c.c. of the sugar broth, grown for two weeks at 20°, was added 95° c.c. of distilled water and two or three drops of phenolphthalein. This was titrated against

$\frac{N}{20}$ NaOH and from the value obtained was subtracted the acidity

of the blank controls titrated at the same time. All tests were made in duplicate, and the final value recorded as the acid or alkali production of the organism is the difference between the average of two titrations of tubes in which it had grown for two weeks and

the average of two blank controls. No determination was made of the rate of acid production as distinguished from this total final acidity, though such observations might be of much interest.

Action upon nitrates.—Data with regard to the reduction of nitrates by the cocci are extremely meager, the presence or absence of this character being recorded in very few of the published descriptions. It seems, however, to have a fair degree of definiteness, and we have included it as a qualitative test in our routine. Each organism was inoculated into a series of 10 tubes of standard nitrate solution. After seven days' growth at 20° the tubes were tested for nitrites and ammonia by the regular method prescribed by the Committee on Standard Methods (1905). The test for nitrates was omitted after it was found that all the cultures, out of a considerable series tested, gave positive results, without exception. The results of the tests for nitrites and ammonia are expressed in the number of tubes which gave positive results, out of the 10 which were tested. In view of the fair constancy of the reaction as observed, we regret that this test was not made quantitative.

Production of indol.—A preliminary examination of some 50 cultures showed no production of indol in any case, and a study of the literature of the cocci indicates that this property is very rare, if it ever occurs, in this group. It was therefore omitted from our routine.

Inhibition of growth by acidity and alkalinity of media.—This character is of considerable importance and warrants careful study, but it is obviously a difficult property to observe in a large series of cultures, and we have not attempted to use it in the present investigation. A preliminary examination of 33 cultures, the results of which are shown in the table, indicated that 1 per cent is the optimum acidity for a majority of these organisms, and that an excess of acidity over this amount is more generally fatal than an alkaline medium.

OPTIMUM REACTION FOR GROWTH AND COLOR PRODUCTION.
NUMBER OF ORGANISMS.

Optimum Reaction	-1.0	-.5	0	+.5	+1.0	+1.5	+2.0
Growth.....	2	4	5	6	9	6	1
Color.....	3	1	0	3	9	6	3

Relation to free oxygen.—The Committee on Standard Methods (1905) recommends that the relation of bacteria to oxygen be studied by the comparison of cultures made under normal, and under anaërobic, conditions. A preliminary study of 50 cultures made in this way led to the belief that such a procedure is unnecessary among the cocci. All but two of the cultures studied showed some growth under anaërobic conditions, but the growth was in most cases meager. It became evident that there are two main types of organisms: those which, like *Streptococcus*, grow only feebly on the surface of aërobic agar, and which grow equally well under anaërobic conditions; and those, like *Sarcina*, which form abundant surface growths under aërobic conditions, and under anaërobic conditions grow feebly like *Streptococci*. In other words, there is little difference between the anaërobic cultures of the cocci. Therefore, for purposes of classification we have considered the study of the aërobic surface growth a sufficient measure of the relation to free oxygen, as well as of general vigor. The five grades recorded under vigor of surface growth correspond fairly well to four grades of aërobiosis, from forms anaërobic and facultatively aërobic, to forms which are strong aërobes.

Temperature relations.—There are two points of special importance which ought to be determined in studying temperature relations, the optimum temperature and the high death-point. The death-point at extremely low temperatures is too indefinite to be attempted, and the extreme limits of growth, although desirable data may be omitted as less important than the other two properties.

For the determination of the optimum temperature we first made a series of preliminary studies by comparing agar cultures grown at 10°, 20°, 37°, 45°, and 56°. We found two cultures growing better at 20°, 18 developed equally well at 20° and 37°, 22 showed an optimum at 37°, two grew equally well at 37° and 45°, and four grew best at 45°. These conclusions refer only to the amount of growth, color production being in most cases most active at 20°. From these results we concluded that the information to be gained by cultures grown below 20° and above 37° would be scarcely commensurate with the labor involved, and we have limited our observation to the comparison of growth and color production at 20° and 37°.

Cultures were grown for this purpose on agar at 20° for two weeks and compared by inspection. Amount of growth and depth of color were recorded in five arbitrary grades as follows: growth or color production, much better at 20°, somewhat better at 20°, equal at the two temperatures, somewhat better at 37°, and much better at 37°.

Thermal death-points were included in the original plan of our experiments and have now been made on 87 cultures. The process used is to inoculate from three- to five-day-old agar cultures into broth tubes brought to the desired temperature in a water-bath heated electrically by a platinum coil, and to expose them for 10 minutes. The tubes are then cooled and incubated at 37° for six days. At the end of that time, streaks are inoculated from the broth tubes in order to make sure by characteristic growth that the organisms originally inoculated are present. Tests are made from 55° up to the point where growth fails. The process is so tedious that we have been unable to complete the work, and must omit this property for the present. The general results so far obtained are as follows:

Thermal Death-Points.
NUMBER OF CULTURES KILLED AT VARIOUS TEMPERATURES.

Temperature	50°	55°	60°	65°	70°	75°	80°
Cultures	2	5	24	17	16	22	1

Pigment formation.—The production of color by the bacteria is not only markedly affected by contemporaneous conditions of cultivation, but may be profoundly modified by selective action or by the effect of antecedent environment. First, of the conditions which temporarily affect the production of color, without modifying the inherent chromogenic power of the organisms, may be mentioned the medium, the presence of free oxygen, and the temperature. In some bacteria, media of low nutritive value, like potato and Nährstoff, appear to favor pigment formation, but with the cocci this is not generally the case. Agar has, on the whole, shown a better development of chromogenesis than any other medium tested. The presence of free oxygen is generally an essential for color production, stab growths being almost invariably lightly colored. We have found a single exception to this rule in a coccus which produces

an orange pigment of much deeper tint in the stab than on the surface. In comparing color at different temperatures we have found, in general, a much better pigment formation at 20° than at 37°. A deep orange growth at the lower temperature may often correspond to a white one at 20°. This effect has been recorded in our routine studies, and will be more fully discussed later with their results as a basis. Besides these temporary modifications of the chromogenic power, the actual color of cultures may be indirectly affected by certain other factors. The general vigor of growth is naturally correlated with apparent depth of color, and the dryness of the atmosphere increases its intensity by evaporating moisture and concentrating the pigment. Both these factors, increase in the total amount of pigment and concentration by evaporation, produce a progressive deepening of color in old cultures.

Even if the temporary conditions of cultivation are quite constant, the chromogenic power of an organism may be modified by its previous history. In thermal death-point observations we have found interesting cases of this sort. Some streaks made from broth cultures which had been exposed to a temperature of 50° or 55° were deeper in color than was the normal for the organism, but in most cases they were much lighter. Sometimes streaks made from a yellow or an orange chromogen after such treatment were almost colorless, although successive transfers generally restored the normal properties. Finally, we have noticed in our work spontaneous variations in chromogenesis such as have been recorded by Neumann (1897), Conn (1900), and Sullivan (1905). The latter authors note that on a plate sown from a single colony there may develop colonies varying appreciably in shade from which selections of the extremes will produce quite distinct types. Neumann records the sudden appearance of widely different strains, as sectors in old and carefully sealed stab cultures. We have observed both phenomena in our cultures, and are inclined to attribute the first, and, more doubtfully, the second, to variation rather than contamination.

In spite of all these facts it is clear that, as the cocci normally occur in nature, chromogenesis is one of their most distinct and significant differences. In any series of plates sown with washings from the skin four well-marked types—red, yellow, orange, and

white—are pretty certain to occur. We have therefore included chromogenesis as one of our routine tests. The variations due to past and present environment are, of course, easily excluded by the maintenance of constant conditions. Our stock cultures were in all cases kept on agar at 20°, and cultures for chromogenesis were grown on that medium, and at that temperature, for two weeks. In order to avoid the apparent differences due to the vigor of growth or to evaporation, a portion of the growth was removed on a loop needle and spread out on white drawing-paper with a rough surface. After drying at the room temperature, the color was compared with an arbitrary standard scheme.

The color chart used for matching these colors was devised after a very careful study of the colors actually found among the Coccaceæ, and includes nine hues ranging from white through lemon-yellow, light cadmium, medium cadmium, lemon-yellow and cadmium orange, red and cadmium orange, to two different combinations of red with lemon-yellow. We have used under each hue, nine different chromas, obtained by successively increasing washes of the hues on white paper. The hue in each case is recorded by a Roman numeral; the chroma, or number of wash, by an Arabic subscript.

Liquefaction of gelatin.—The liquefaction of gelatin, like the property of pigment production, has been shown to be subject to fluctuating variations. Conn (1900) was able by selection to obtain from a single culture of a milk coccus a rapidly liquefying form, and one with almost no peptonizing power. Smith (1900) records similar experiences with colon bacilli and forms of *B. proteus*. There appears to be little correlation between liquefaction and any other power, since it is so common in widely separated groups of bacteria to find organisms differing in this respect, while identical in all other properties.

In studying liquefaction we have determined only the amount of the action exerted by each organism. The shape of the liquefaction in the stab culture has been shown by Whipple (1902) to vary within the widest limits, with slight differences in the character of the medium, and the Committee on Standard Methods (1905) has omitted this character from its list.

For determining the amount of liquefaction we have used the

method suggested by Clark and Gage (1905), which consists in inoculating gelatin tubes of 10 mm. diameter by spreading a suspension of the culture over the surface. Liquefaction proceeds in a strati-form fashion, and its amount may be read off in centimeters. With such a method one may determine the rapidity of liquefaction either

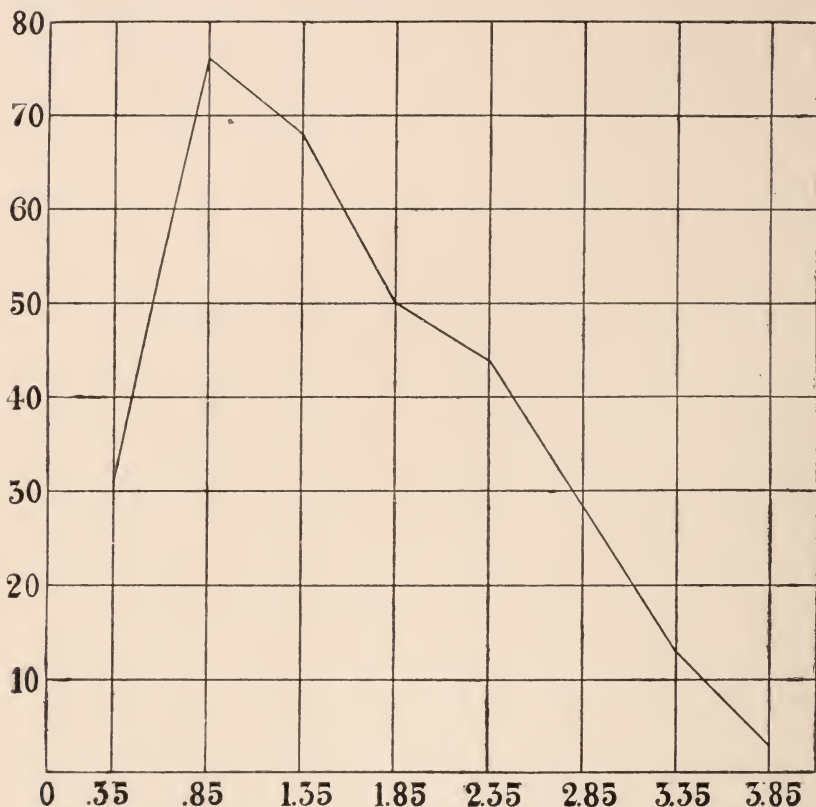


FIG. 4.—Liquefaction of gelatin by 314 cocci. Abscissae, liquefaction in cm. Ordinates, number of cultures.

by the number of days required to reach a fixed point, or the final amount of liquefaction. In general, these two values are pretty closely correlated, but in a preliminary study we found that the final differences are somewhat sharper as well as easier to record. We have therefore adopted as our routine measure of liquefying power the depth of liquefaction after 30 days.

Supplementary tests.—Many other tests than those mentioned are sometimes used in bacterial diagnosis, but none have seemed suited to the present study. The questions of pathogenicity and agglutinative power are so shrouded in confusion as to be unpromising. Meyer (1902) considered serum reactions of diagnostic value among the streptococci, and Kolle and Otto (1902), and Otto (1903), obtained good results with the staphylococci. On the other hand, Aronson (1903), Fischer (1904), and Kerner (1905), after very thorough investigations, came to the conclusion that these properties among the streptococci are so erratic as to be quite valueless in systematic work. From a general survey of the literature of the group, it seems probable that the properties connected with infection and immunity are likely to be too easily modified to prove helpful in classification.

The test for liquefaction of starch is one which it seems logical to include with those which show the relation of an organism to gelatin and the sugars; and we made some experiments with the starch media introduced by Smith (1905). It appeared that certain cocci did exert an amylolytic action and the study of this character would probably prove of considerable interest. It has been omitted for the present, for lack of time.

III. RESULTS OF THE INVESTIGATION.

The characters observed and the terms in which they are recorded may be summarized as follows:

1. *Habitat.*—Recorded as 1 (diseased conditions); 2 (normal body); 3 (water); 4 (earth); or 5 (air). The significance of these various habitats has been more fully discussed above. It should be noted that Group 5 includes certain laboratory cultures whose origin was unknown.

2. *Grouping of cells and dimensions.*—Observed in stained preparations, made from 20° agar cultures less than five days old. Grouping recorded as 1 (packets present); or 2 (packets not present). Extreme dimensions recorded in micromillimeters to the nearest 10th.

3. *Relation to Gram stain.*—Observed on two occasions on 20° agar cultures less than five days old. Treated with anilin-oil-gentian-

violet for one and one-half minutes; Gram's solution, one and one-half minutes; 95 per cent alcohol, three minutes. Counterstained with Bismarck brown for one-half minute. Reaction recorded as — (decolorized in both tests); \pm (stained once and decolorized once); or + (stained in both tests).

4. *Vigor of surface growth on agar streak after 14 days at 20°.*—Recorded as 1 (very faint); 2 (meager); 3 (good); 4 (abundant); or 5 (very heavy).

5. *Amount of acid produced in 2 per cent dextrose broth after 14 days at 20°.*—Determined by titration against $\frac{N}{20}$ NaCl in the cold with phenolphthalein as an indicator. Recorded value is the difference between inoculated tubes and sterile controls, expressed in cubic centimeters to nearest 10th.

6. *Amount of acid produced in 2 per cent lactose broth.*—Same conditions as under 5.

7. *Formation of nitrites in nitrate solution.*—Recorded value is the number of tubes giving positive test for nitrites out of a series of 10, grown for seven days at 20°.

8. *Formation of free ammonia in nitrate solution.*—Same method as under 7.

9. *Comparative growth after 14 days' growth on agar streak at 20° and 37°, respectively.*—Recorded as 1 (much more vigorous at 20°); 2 (more vigorous at 20°); 3 (equal); 4 (more vigorous at 37°); or 5 (much more vigorous at 37°).

10. *Chromogenesis.*—Hue and chroma of pigment produced on agar at 20° after 14 days, determined by comparison with color scheme as described later.

11. Depth in cm. of gelatin liquefaction in tube of 1 mm. diameter after 14 days at 20°.

It would be well to extend this series of tests by a study of the cell-grouping in broth, motility, fission on the agar block, fermentation of saccharose, effect of acid and alkaline media, and the thermal death-point.

I. HABITAT.

The distribution of the cultures isolated among the various habitats was as follows: (1) diseased conditions, 59; (2) normal body,

170; (3) water, 95; (4) earth, 67; (5) air, 109. It is probable that this deviates from a representative sampling of the cocci in nature by laying too great stress on the saprophytic forms. It is difficult to find cocci at all in earth and water, whereas they are present on the surfaces of the body in enormous numbers. The majority of this group appear to be parasitic or semiparasitic in habit. At the same time, the fairly equal weight given to the saprophytic forms helps to bring out the differences between the two main groups, those living in or on the animal body (1 and 2), and those living in the outer world (3, 4, and many of 5).

We have prepared tables to show the distribution of each character among various habitats, and the relations shown are so suggestive as to warrant rather full discussion. In Table 1 is given the correlation between habitat and cell-grouping, and it is at once evident that the sarcinæ occur in greater proportion outside than inside the body.

In this and succeeding tables the figures represent the number of cultures showing each combination of characters out of the 500 studied.

TABLE 1.
CORRELATION BETWEEN HABITAT AND CELL-GROUPING.

	Diseased Conditions	Normal Body	Water	Earth	Air
No. packets.....	44	145	45	33	78
Packets.....	15	25	50	34	31

Whereas packets are more abundant in earth and water, the other forms—chains, plates, and irregular groups without sarcinæ—make up two-thirds or more of the parasitic forms.

TABLE 2.
CORRELATION BETWEEN HABITAT AND GRAM STAIN.

Gram Stain	Diseased Conditions	Normal Body	Water	Earth	Air
-	14	12	46	37	36
±	17	50	29	18	32
+	28	108	20	12	41

The distribution of our cultures according to their relation to the Gram stain brings out a similar condition. The cultures giving

a consistent positive reaction make up far more than half the total among the parasitic forms from the first two habitats, and less than one-fourth of the forms from water and earth. The air cultures in almost all cases exhibit an intermediate relation, as would be expected, since they must contain forms from both sources. A positive reaction to the Gram stain is evidently closely correlated with life in and on the animal body.

TABLE 3.
CORRELATION BETWEEN HABITAT AND SURFACE GROWTH.

Surface Growth	Diseased Conditions	Normal Body	Water	Earth	Air
Very faint.....	0	16	3	0	0
Meager.....	1	15	2	2	1
Good.....	23	98	38	24	32
Abundant.....	27	34	38	31	60
Very heavy.....	8	7	14	10	16

The abundance of surface growth also varies with the habitat. Very faint and meager growths are fairly abundant in the forms from the surfaces of the body, as would be expected, since our culture media are unfavorable for the more strictly parasitic forms. On the other hand, a majority of the earth and water cocci show abundant or very heavy surface growths. The air forms are characterized by particularly abundant development, as would naturally be expected, since only the vigorous cells probably survive drying and dispersal through the air.

TABLE 4.
CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
0.0 and alkaline.....	12	12	39	28	20
0.1-0.2.....	7	33	22	24	24
0.3-0.6.....	20	82	19	10	25
0.7-2.0.....	17	38	12	5	34
2.0 and over.....	3	5	3	0	6

TABLE 5.
CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
-0.2 and more alkaline.....	6	12	9	10	13
-0.1 and 0.0.....	23	37	62	42	40
0.1-0.4.....	15	64	16	11	32
0.5-1.4.....	11	49	5	4	19
1.5 and over.....	4	8	3	0	5

In examining Tables 4 and 5, which show the fermentative power of dextrose and lactose broth, the fundamental difference between the parasitic and saprophytic cocci is again made evident. In the first two groups, from the animal body, over two-thirds of the cultures produce more than 0.3 per cent of normal acid; while among the earth and water forms two-thirds of the organisms form less than this amount. With lactose the same law holds. Two-thirds of the cocci from the normal body produce acid in lactose, against less than one-third of the water and earth forms. The air cultures show an intermediate relation.

TABLE 6.
CORRELATION BETWEEN HABITAT AND REDUCTION OF NITRATES.

	Diseased Conditions	Normal Body	Water	Earth	Air
No reduction.....	44	147	75	44	72
Nitrites formed.....	10	19	13	10	18
Ammonia formed.....	7	7	8	13	30

The property of nitrate reduction does not appear to be related to habitat in any such direct way as the other characters studied. The air cocci, however, show a peculiarity of considerable interest, nitrite formation being common, and ammonia formation very common, among them.

TABLE 7.
CORRELATION BETWEEN HABITAT AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum	Diseased Conditions	Normal Body	Water	Earth	Air
20°.....	9	11	29	23	11
20° or 37°.....	36	112	56	42	89
37°.....	14	47	10	2	9

While a majority of the cultures studied grow indifferently at 20° or 37°, it appears from Table 7 that among the parasitic forms a fair proportion are favored by the body temperature, while more of the earth and water forms develop best at 20°. With regard to the optimum temperature for color formation, no definite relation with habitat appears, except as involved in the double relation between chromogenesis and habitat, and chromogenesis and the optimum temperature for color formation. These figures are therefore omitted.

TABLE 8.
CORRELATION BETWEEN HABITAT AND CHROMOGENESIS.

Chromogenesis	Diseased Conditions	Normal Body	Water	Earth	Air
White.....	4	17	5	1	13
Yellow.....	33	37	76	50	58
Orange.....	21	116	6	10	28
Red.....	1	0	8	6	10

Table 8 brings out some of the most definite relations yet considered, between habitat and chromogenesis. It is evident that the white and orange forms are largely parasitic, and the yellow and red forms as distinctively saprophytic. More than half of the white and more than two-thirds of the orange chromogens come from the first two habitats, while only one-third of the yellow forms have such an origin. The distribution of the red pigment-formers is even more notably saprophytic. Only one culture out of 25 occurred among the 229 cultures from the body.

TABLE 9.
CORRELATION BETWEEN HABITAT AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Diseased Conditions	Normal Body	Water	Earth	Air
0.0.....	13	68	43	26	36
0.1-1.5.....	24	36	42	30	45
1.6 and over.....	22	66	10	11	28

The table for habitat and gelatin liquefaction (Table 9) shows this property occurring among the earth and water forms to a less degree than among the parasitic cocci. This fact, and the fact that the parasitic forms are high acid-producers, as noted above, are of practical significance in connection with the bacteriological analysis of water. It has long been suspected that acid production and gelatin liquefaction were associated with intestinal organisms, and we have here a measure of the truth of this proposition, in the case of the cocci at least. From the food conditions which obtain in the alimentary tract, and to a less extent on the outer surfaces of the body, it is natural that these properties should be highly developed.

The forms from Habitat II (the surfaces of the normal body) group themselves most abundantly at two extremes, 68 being non-

liquefiers and 66 active liquefiers, while only 36 occupy the intermediate position, which is of most frequent occurrence in all the other habitats. It is probable that this may be accounted for by the presence, in Habitat II, of two distinct series—the white and colorless forms, which, as we shall see later, are non-liquefiers, and the orange forms, which peptonize strongly.

From a general survey of our habitat studies it is evident that the forms from the body exhibit quite different characteristics from those of the water and earth cocci. The parasitic forms generally react positively to the Gram stain, give only fair surface growths on the surface of artificial media, produce acid in dextrose and lactose, grow best at 37°, produce no pigment or a white or an orange pigment, and liquefy gelatin. The saprophytes, on the other hand, are more apt to occur in packets, to be Gram-negative, to grow abundantly on artificial media, best at 20°, to produce yellow and red pigments, and to exert little action on sugars and gelatin. The air cocci are generally intermediate in character between the two groups, but show special powers of nitrate reduction.

2. GROUPING OF CELLS, AND DIMENSIONS.

The cocci, as noted above, were divided into two classes only, according to the character of the cell aggregates; 155 cultures showed the packets or sarcina-grouping, and 345 did not.

TABLE 10.
CORRELATION BETWEEN CELL-GROUPING AND GRAM STAIN.

Gram Stain	Irregular Groups and Chains	Packets
—.....	75	70
±.....	98	48
+.....	172	37

We have pointed out above that packets are most abundant among the saprophytic cocci of the earth and water. Table 10 shows the relation between cell-grouping and the Gram stain, clearly indicating that the packets tend to be Gram-negative, while a majority of the other forms give a positive reaction.

Table 11 shows a distinct correlation between cell-grouping and the vigor of surface growth. A large majority of the non-packet-

forming organisms produce only very faint, meager, or good growths while a large majority of the sarcinæ form abundant or very heavy

TABLE 11.
CORRELATION BETWEEN CELL-GROUPING AND SURFACE GROWTH.

Surface Growth	Irregular Groups and Chains	Packets
Very faint.....	18	1
Meager.....	16	5
Good.....	169	46
Abundant.....	132	58
Very heavy.....	10	45

growths. The fact that the packet-forms flourish on artificial media should naturally result from their saprophytic origin.

TABLE 12.
CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
0 and under.....	58	53
0.1-0.2.....	56	54
0.3-0.6.....	128	27
0.7-2.0.....	87	20
2.0 and over.....	16	1

TABLE 13.
CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
-0.2 and more alkaline	38	11
0.1 and 0.0.....	113	91
0.1-0.4.....	104	35
0.5-1.4.....	72	16
1.5 and over.....	18	2

A marked correlation between the packet-forming organisms (presumably saprophytic) and the fermentation of sugars is manifested in Tables 12 and 13. Taking Table 12 as an illustration, there will be found to be about 60 per cent of the packet-formers producing alkali, or, at most, fermenting dextrose but slightly (to 0.2)—almost none of the organisms occurring in the class of highest acid producers. Conversely, 70 per cent of the organisms which do not form packets produce acid from 0.3 up to the highest amount.

The power to reduce nitrates appears with about equal regularity in both our morphological groups.

TABLE 14.
CORRELATION BETWEEN CELL-GROUPING AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Irregular Groups and Chains	Packets
20°.....	40	43
20° or 37°.....	237	98
37°.....	68	14

A slight but distinct correlation appears between grouping and optimum temperature for growth. In each case most of the cultures grow at 20° or 37° indifferently; but a fair proportion of the more saprophytic sarcinæ show better development at 20°, while among the other forms more find an optimum at 37° than at 20°. With regard to the optimum temperature for color formation there is a slight difference, only two-fifths of the sarcinæ showing more chromogenesis at 20° than at 37°, against one-half of the other cultures. This, as we shall see later, is probably connected with a difference in chromogenesis.

TABLE 15.
CORRELATION BETWEEN CELL-GROUPING AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Irregular Groups and Chains	Packets
0.0.....	127	59
0.1-1.5 cm.....	110	67
1.6 and over.....	108	29

Table 15, for the relation of gelatin liquefaction to morphology, shows only that the highest grades of liquefaction are somewhat less numerous among the packets than in the other group.

The results obtained with regard to the size of the individual cell were much less suggestive than the facts concerning cell-grouping. Dimensions were measured in all cases on the stained specimens, and were recorded independently on at least two occasions. The attempt was made to note in each case the extreme diameters observed, and the values finally adopted represent the average between the recorded extremes. Individual cells ranged from 0.1 to 2.0 μ . With the packets it was found impossible to determine the maximum size of the single cell, on account of the frequent occurrence of small, recently formed packets which stained as a whole like one cell. We never felt certain that what appeared like a large cell was not

really a group of eight small ones. The sarcinæ in general showed quite small individual units, 0.1 or 0.2μ as a rule, with no constant deviations. The packets are therefore entirely omitted from the consideration of dimensions.

The average sizes of the 345 cultures, not occurring in packets, are grouped in convenient classes in Table 16 and plotted in Fig. 1.

TABLE 16.
DIMENSIONS OF 345 COCCI.

Size, average, μ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Number of cultures	22	82	113	60	40	12	7	6	1	2

The sizes of the cocci studied are evidently distributed on a fairly normal curve of frequency. The mode is at 0.3μ and the curve is markedly skew, with infinite extension toward the larger sizes. The important practical point is that the forms measured appear to behave like a fairly homogeneous series varying about a single mode.

We have made tentative calculations of correlation between cell dimensions and other characters, with almost entirely negative result. The only property showing any relation is that of gelatin liquefaction.

TABLE 17.
CORRELATION BETWEEN CELL DIMENSIONS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Maximum Size 0.3μ and under	Maximum Size over 0.3μ
0.0	47	78
0.1-1.5	65	47
1.6 and over	60	39

An appreciable inverse correlation is shown between the size of the cell and the rate of gelatin liquefaction, the smaller cocci liquefying most readily.

3. GRAM STAIN.

We have pointed out above that the reaction of the cocci to the anilin-oil-iodin stain is a variable character, many forms showing a positive reaction on one occasion and a negative reaction when next tested. Nevertheless the test, variable as it is, shows quite constant relations to other characteristics; and we feel convinced that among the cocci where all characters are more or less fluctuating any prop-

erty which on the average shows a definite correlation with other properties has systematic significance.

Of the cocci studied, 145 showed in two tests a Gram negative reaction on both occasions, and 209 two positive reactions, while 146 were once stained and once decolorized. Grouped thus in three divisions, we have seen that the positive reaction is characteristic of the parasitic forms, while saprophytic forms and packets tend to be Gram negative. With surface growth only an insignificant relation appears, the less richly growing forms showing a slightly higher proportion of positive reactions.

TABLE 18.
CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
0.0 and over.....	49	44	18
0.1-0.2	48	29	33
0.3-0.6	35	45	75
0.7-2.0	12	26	69
2.1 and over	1	2	14

TABLE 19.
CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
-0.2 and more alkaline	20	14	15
-0.1 and 0.0	72	82	50
0.1-0.4	34	24	81
0.5-1.4	19	20	40
1.5 and over	0	6	14

The relation between the Gram reaction and the fermentation of carbohydrates is a surprisingly close one. Each line in Tables 18 and 19 showing the distribution of organisms among the grades of acidity forms a regular curve. In each case the mode of the Gram-negative cultures occurs at the neutral point, and that of the Gram-positive cultures at a moderately high acidity, with the doubtful cultures showing an intermediate relation.

TABLE 20.
CORRELATION BETWEEN GRAM STAIN AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Gram Negative	Gram Variable	Gram Positive
20°	34	29	20
20° or 37°	103	89	143
37°	8	28	46

With nitrate reduction and the optimum temperature for chromogenesis the Gram reaction shows no special relations. With optimum growth temperature, on the other hand, Table, 20 shows a distinct connection. As always, most of the cultures grow equally at both temperatures. Among the decolorized cultures, however, a fair proportion grow best at 20°, while with the positive forms 37° is most favorable. Such a relation would, of course, be expected from the generally saprophytic habit of the negative forms.

The liquefaction of gelatin does not show any distinct relation to the Gram reaction. On the whole, therefore, we may conclude that the cocci which decolorize by Gram are generally earth and water forms, which notably fail to ferment sugars, and which grow best at 20°. The marked correlation with the power of acid production, in the absence of other equally marked relations, seems to invite further study of the physiological basis of these properties.

4. SURFACE GROWTH.

The cocci studied were divided into five groups according to the vigor of surface growth on agar. The first group, of "very faint" growths, includes 19; the second group, of "meager" growths, includes 21 forms; "good" and "abundant" growths occur 215 and 190 times, respectively; and 55 cocci show "very heavy" growths.

TABLE 21.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0 and alkaline	3	2	42	41	23
0.1-0.2	1	3	41	51	14
0.3-0.6	3	11	76	54	12
0.7-2.0	5	4	53	39	5
2.0 and over	7	1	3	5	1

TABLE 22.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
-0.2 and more alkaline.....	2	3	18	22	5
-0.1 and 0.0.....	2	5	80	87	30
0.1-0.4.....	5	8	65	40	11
0.5-1.4.....	2	4	47	27	8
1.5 and over	8	1	5	5	1

We have seen above that the fainter growths are characteristic of the parasitic forms, and the heavier ones of the earth and water cocci. The heavier growths are more common among the packets than with other cell groupings.

In comparing the vigor of surface growth with the fermentation of carbohydrates, a distinct relation appears at the ends of the scale, although the bulk of the growth, under the headings "good" and "abundant," exhibit uniform characteristics. The "very faint" growths, which denote members of the genus *Streptococcus*, as previously defined, are associated with a maximum of acid production falling in the highest acidity class in each sugar table. On the other hand, the "very heavy" growths are mainly forms which fail to act on either sugar.

TABLE 23.
CORRELATION BETWEEN SURFACE GROWTH AND NITRATE REDUCTION.

	Very Faint	Meager	Good	Abundant	Very Heavy
No reduction	19	18	173	133	39
Nitrites formed	0	2	25	38	5
Ammonia formed	0	1	23	29	12

Surface growth and nitrate reduction show a suggestive relation. Among the very faint growths of the *Streptococcus* type no reduction occurs, and almost none among the "meager" forms. The "good" and "abundant" groups show an increasing proportion of reducing organisms, and the "very heavy" group shows many ammonia-formers and a fair proportion of nitrite production.

TABLE 24.
CORRELATION BETWEEN SURFACE GROWTH AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0.....	19	14	63	80	10
0.1-1.5.....	0	6	84	62	25
1.6 and over	0	1	68	48	20

With gelatin liquefaction there exists the same group correlation manifest for the other characters. The "very faint" group shows not one liquefier, and the "meager" group very few, while the more vigorous forms exhibit a more even distribution.

In general, our study of surface growth brings out two distinct

groups of organisms. The first group, including the forms with faint or meager surface development, corresponds to the genus *Streptococcus* as defined above. It is sharply characterized by high acid production and the absence of gelatin liquefaction or nitrate reduction. Cocci of this type are characteristically parasitic, and very rarely show the sarcina grouping. On the other hand, the more vigorous forms are generally saprophytic, and frequently show packets. They ferment sugar slightly or not at all, and often reduce nitrates and liquefy gelatin.

5. FERMENTATION OF CARBOHYDRATES.

In measuring the amount of acid produced in dextrose and lactose broth, two check determinations were made in each case, and the figure finally recorded was the average of these two determinations. The correspondence between the two tubes was generally close. From 150 cases for each sugar we have calculated the probable error of a single observation, and find it only ± 0.043 for dextrose and ± 0.036 for lactose. Since our readings were only taken to 10ths of a c.c., it is evident that even a single determination would be sufficiently accurate for any long series.

The general results of the titrations are shown in Table 25 and in Fig. 2. It will be noticed that with both acids the organisms are ranged with fair regularity about a single mode. The majority of the cultures studied produce an acidity of 0.1-0.2 per cent normal in dextrose, and fail to ferment lactose at all. In either case a few cultures only show an alkaline reaction, and with lactose less than half of the organisms form acid, giving the curve for that sugar a very acute form. The curve for dextrose falls off much more slowly, and shows slight secondary modes at acidities of 0.5-0.6 per cent normal and 1.3-1.4 per cent normal. Finally, both curves show an extraordinary extension in the direction of the higher acidities. It will be noticed that for each sugar several of the highest reactions, ranging from 3 to nearly 10 per cent normal, have been omitted from the chart. We may fairly consider the fermentation of the two carbohydrates together, since, as shown in Table 26, they are very closely correlated. The amount of acid produced in lactose broth is almost always less than that in dextrose broth, but the two vary together.

TABLE 25.
ACID PRODUCTION IN SUGAR BROTHS.

Acidity Produced (Per Cent Normal)	Number of Cultures in Each Group							
	-0.9-0.8	-0.7-0.6	-0.5-0.4	-0.3-0.2	-0.1-0.0	0.1-0.2	0.3-0.4	0.5-0.6
Lactose	1	1	7	41	204	86	52	44
Dextrose	4	9	98	110	80	76

	0.7-0.8	0.9-1.0	1.1-1.2	1.3-1.4	1.5-1.6	1.7-1.8	1.9-2.0	2.1-2.2	2.3-2.4
Lactose	23	10	7	4	5	2	1	3	2
Dextrose	45	28	12	13	6	2	0	4	1

	2.5-2.6	2.7-2.8	2.9-3.0	3.1-3.2	3.3	4.0	4.3	4.6	4.7	5.9	8.2	8.6	9.7
Lactose	1	1	1	..	2	1	..	1
Dextrose	3	2	0	2	..	1	1	..	1	1	1

TABLE 26.
CORRELATION BETWEEN FERMENTATION OF DEXTROSE AND LACTOSE BROTHS.

Dextrose Acid Production (Per Cent Normal)	Lactose -0.2 and more alkaline	Lactose -0.1 and 0	Lactose 0.1-0.4	Lactose 0.5-1.4	Lactose 1.5 and over
0 and alkaline.....	19	72	15	4	1
0.1 and 0.2.....	11	57	29	13	0
0.3-0.6.....	11	47	55	40	1
0.7-2.0.....	9	26	35	29	6
2.0 and over.....	0	2	1	2	12

We have noted before that the power of carbohydrate fermentation is specially characteristic of the parasitic cocci, and of those which do not show the packet grouping. The high acidities are also correlated with a positive reaction to the Gram stain and with a faint surface growth on artificial media.

TABLE 27.
CORRELATION BETWEEN FERMENTATION OF DEXTROSE BROTH AND NITRATE REDUCTION.

Dextrose Acid Production (Per Cent Normal)	No Reduction	Nitrite Found	Ammonia Found
0 and alkaline	75	13	16
0.1 and 0.2	86	11	16
0.3-0.6.....	115	28	18
0.7-2.0.....	90	18	15
2.0 and over	16	0	0

Table 27, for the relation between nitrate reduction and acid formation in dextrose broth, shows only that the class of strong

acid-formers fail entirely to form nitrites and ammonia. A correlation table for lactose, which we have not thought it necessary to quote here, shows a similar relation. The high acid-formers, it may be remembered, belong to the genus *Streptococcus*, with its weak power of growth on artificial media.

Our tables of the correlation between carbohydrate fermentation and optimum temperature fail to show any striking coincidences. There is an appreciable tendency for the higher acid-formers to grow better at 37°, and for the alkaline or neutral forms to grow better at 20°; but we have not considered this important enough to warrant the reproduction of the tables.

TABLE 28.
CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1-1.5 cm.)	Gelatin Liquefied (1.6 cm. and over)
0 and alkaline.....	38	61	12
0.1 and 0.2.....	45	42	23
0.3-0.6.....	47	37	72
0.7-2.0.....	43	36	27
2.0 and over.....	13	1	3

TABLE 29.
CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1-1.4 cm.)	Gelatin Liquefied (1.5 cm. and over)
-0.2 and more alkaline	20	19	11
-0.1 and 0.....	91	86	27
0.1-0.4.....	49	46	43
0.5-1.4.....	13	25	50
1.5 and over.....	13	1	6

The relation between the organisms which ferment the sugar broths and liquefy gelatin is shown in Tables 28 and 29. These tables may be considered together, as they reveal practically the same law. The relation between acid production and gelatin liquefaction is evidently a somewhat complex one. The forms which fail to ferment carbohydrates for the most part exhibit a moderate amount of liquefaction. Next comes a group of the moderate acid-producers which liquefy most actively. Finally, the highest acid-formers are mostly non-liquefiers. We shall get more light on these three groups when we come later to consider the classes of the cocci according to their chromogenesis.

To our conception of the non-acid-forming cocci as typically saprophytic organisms frequently occurring in packets and usually Gram-negative, we may add the property of moderate, but not very active, liquefaction of gelatin. The very high acid-producers are generally parasitic, do not show sarcinæ, stain by Gram, grow faintly on agar, and fail to reduce nitrates or liquefy gelatin. Between these two groups is a third type which forms a moderate amount of acid and produces the most active liquefaction of gelatin.

6. REDUCTION OF NITRATES.

As described above, the tests for nitrate reduction were made in parallel in 10 tubes, and a marked variation was found in the individual tubes, as shown in Table 30. This is perhaps to be expected, since the development of bacteria in such a nutrient medium as nitrate solution must be subject to many chance variations in the number and vigor of the organisms inoculated.

TABLE 30.
REDUCTION OF NITRATES.

Number of Tubes Showing Positive Tests.....	1	2	3	4	5	6	7	8	9	10
Nitrites.....	27	14	8	5	11	5	11	7	12	24
Ammonia.....	30	26	21	15	16	10	2	11	4	22

Table 30 shows a considerable number of cultures yielding positive results in one or two out of the 10 tubes tested, less in from four to seven of the tubes, and more again giving check results in all 10 tubes. In order to compare this property with others, it was necessary to distinguish between positive and negative cultures, and we have therefore considered the test to be positive when five or more of the tubes showed some reduction. The cultures then grouped themselves into three classes—one a large one, of those organisms which did not have the property of nitrate reduction, and the two smaller classes, in which were those which formed nitrites and those which formed ammonia.

Table 31 shows a somewhat surprising lack of correlation between the formation of the two reduction products for which we have made test. Only 17 cultures showed both nitrites and ammonia in five

or more of the 10 tubes, while 48 cultures formed nitrites alone, and 53 cultures ammonia alone, according to the same standard. It seems improbable that in the latter case nitrites had been formed and entirely reduced to ammonia. We are inclined rather to conclude

TABLE 31.
CORRELATION BETWEEN NITRITE FORMATION AND AMMONIA FORMATION.

	Nitrites +	Nitrites -
Ammonia +	17	48
Ammonia -	53	382

that two different types of reduction exist, in one of which ammonia is produced directly.

As regards correlation with other properties, we have seen that the production of nitrites, and still more notably that of ammonia, is especially characteristic of the cocci isolated from the air. It is, of course, possible that this may be indirectly connected with the fact that forms which have survived drying and dispersal through the air must be particularly well adapted to conditions which obtain in the nitrate solution. A similar law is apparently manifest in the striking relation to vigor of surface growth. The power of forming both reduction products increases progressively with the richness of surface growth, being entirely absent in the "very faint" class. No relation appears between nitrate reduction and optimum temperature, and the only other correlation to be considered is that with gelatin liquefaction shown in Table 32.

TABLE 32.
CORRELATION BETWEEN NITRATE REDUCTION AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (in cm.)	Nitrates Not Reduced	Nitrites Formed	Ammonia Formed
3.0.....	152	26	13
1.-1.5.....	131	27	25
1.6 and over	99	17	27

Table 32 shows the usual large proportion of organisms which do not exert nitrate reduction, but it may be noticed that some 30 per cent of the liquefiers reduce nitrates, against only 20 per cent of the non-liquefiers. Again, only 6 per cent of the non-liquefiers, against 16 per cent of the liquefiers, form ammonia.

7. OPTIMUM TEMPERATURE.

We have divided the cocci into five groups, according to their optimum growth temperature. Forty-one cultures gave "much better," and 42 "better," growth at 20°; 335 developed "equally" at both temperatures; 57 grew "better," and 25 "much better," at 37°.

We have classed together the first two and last two groups. In making the tables it was more convenient to have fewer groups, and quite as accurate, since the main distinctions (and those not very rigid) are shown in "better growth at 20°" or "better at 37°," and "equal" growth at both temperatures.

We have already noted the correlation between optimum temperature and habitat, the parasitic forms growing best at 37°, and the saprophytic forms at 20°, when any difference appears. The sarcinæ belong notably to the second class, as do the Gram-positive cultures. These are the only correlations which have so far appeared.

We have been somewhat surprised not to find special correlation between the optimum temperature for growth and that for color production; but no such correlation appears. With gelatin liquefaction also no definite relation appears.

We have observed also the effect of the body and room temperature upon color production, but without important results. Of the cocci studied, 69 showed a very much higher chromogenic power at 20° than at 37°; 169 showed more color, but not so much more, at the lower temperature; in 245 cases no difference appeared, while 13 cultures showed more, and 14 cultures much more, pigment at 37°. We have calculated correlation tables for all the various characters studied, but in no case did any constant relation appear, except, as noted later, in connection with the kind of chromogenesis.

8. CHROMOGENESIS.

As noted above, chromogenesis was determined by matching the pigment dried on white paper against a color chart prepared after a thorough study of the colors actually found among the cocci. This chart included nine hues, designated by Roman numerals, corresponding to the pigments noted below the figure. Under

each hue were nine different chromas, indicated by Arabic numerals, each figure indicating the number of washes of pure color added to obtain the particular chroma.

The distribution of the organisms studied under these different colors is indicated in Fig. 3, where the vertical columns indicate the hues from I (white), through the yellows (II-IV), the oranges (V and VI) to the reds (VII-IX), and the horizontal columns the successive chromas.

On inspection of this chart, bearing in mind the colors signified, there appear at once four modes—one occurring in each chief color.

That for the white falls at I₁, for the yellows at IV₃, for the oranges at VI₈, and for the reds at VII₈. These are not, of course, the points of intensest color, but of the most concentrated distribution. The evident clustering of the individuals around a mode, and the consequent falling-away of the numbers between the modes, suggest a variation from an ancestral center. Like most living things governed by an evolutionary law of gradual change, the hues grade so gently into each other that the exact placing of lines of division must be arbitrary. We have, however, assumed four divisions as a basis for our work, and separated them at the lowest points between the modes, as shown by the heavy black lines in the chart, which divide the group of bacteria producing a white pigment from that which produces a yellow, the yellow from the orange, and the orange from the red. The striking correlations obtained between chromogenesis and other properties have convinced us that this division was a sound and natural one. It should be noted, however, that the division of the "white" chromogens includes two sub-groups—the true white pigment-formers and the forms which produce such a faint surface growth that no distinct color is apparent.

We have omitted the consideration of chromogenesis from our correlation tables, except that for habitat, preferring to consider all chromogenic relations under one head. It will appear, on inspection of the following tables, that this character is really the key by which most of the other correlations may be explained, and is perhaps the most important single factor in the systematic grouping of the Coccaceæ.

It was shown under "Habitat" that the white and orange chromo-

gens were chiefly parasites, the yellow and red chromogens chiefly saprophytic forms. The same distinction is shown in Table 33 with regard to cell-grouping. The white and orange cocci only rarely,

TABLE 33.
CORRELATION BETWEEN CHROMOGENESIS AND CELL-GROUPING.

Cell-Grouping	White	Yellow	Orange	Red
Irregular Groups and Chains.....	33	134	163	15
Packets.....	7	120	18	10

the latter very rarely, show packets. The yellow and red forms, on the other hand, show the sarcinæ-grouping almost half the time.

TABLE 34.
CORRELATION BETWEEN CHROMOGENESIS AND GRAM STAIN.

Gram Stain	White	Yellow	Orange	Red
-.....	6	100	15	15
±.....	9	84	46	7
+.....	25	61	120	3

The reaction to the Gram stain exhibits a still more perfect correlation. Among the whites and oranges (the parasitic forms) positive Gram reactions predominate, and negative ones are rare. Among the saprophytic yellows and reds conditions are symmetrically reversed.

TABLE 35.
CORRELATION BETWEEN CHROMOGENESIS AND SURFACE GROWTH.

Surface Growth	White	Yellow	Orange	Red
Very faint.....	14	3	2	0
Meager.....	3	6	12	0
Good.....	7	100	107	1
Abundant.....	13	95	58	24
Very heavy.....	3	50	2	0

A comparison of the general vigor of growth shows that each color has its own relation. Among the white forms, two maxima appear, one under "very faint" growth and one under "abundant" growth. This is because this group is a compound one, including forms which give a really white growth abundant in amount, and the feebly growing streptococci which are classed here, although they produce no pigment at all. The yellow and orange chromo-

gens show maxima under the "good" growth, almost all the "very abundant" growths belonging to the former class. The red forms are almost all of one type—the "abundant."

TABLE 36.
CORRELATION BETWEEN CHROMOGENESIS AND DEXTROSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
0.0 and alkaline.....	5	94	7	5
0.1-0.2.....	7	72	24	7
0.3-0.6.....	5	50	92	9
0.7-2.0.....	15	35	53	3
Over 2.0.....	8	3	5	1

TABLE 37.
CORRELATION BETWEEN CHROMOGENESIS AND LACTOSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
-0.2 and more alkaline.....	3	33	9	5
-0.1 and 0.0.....	8	141	39	16
0.1-0.4.....	12	59	64	3
0.5-1.4.....	6	18	63	1
1.5 and over.....	11	3	6	0

The correlations between chromogenesis and the fermentation of the sugars are singularly perfect. The white forms in each case show two maxima, one corresponding to the true white chromogens, the second, at a higher acidity, to the colorless streptococci. The latter include a majority of the strongest acid-producers in each case. The other types show for each sugar a regular and characteristic curve, the elements from which the complex curve in Fig. 2 is made. The yellow forms show for each sugar a mode at the neutral point. The orange chromogens, on the other hand, are most abundant at an intermediate grade of acidity, most of them producing 0.3-0.6 per cent acidity in dextrose broth, and 0.1-0.4 per cent in lactose broth. The red forms show the same relation as the orange forms toward dextrose, while in lactose broth they resemble the yellow chromogens, producing in most cases no change of reaction.

TABLE 38.
CORRELATION BETWEEN CHROMOGENESIS AND NITRATE REDUCTION.

	White	Yellow	Orange	Red
No reduction.....	35	197	137	13
Nitrites produced.....	3	30	25	12
Ammonia produced.....	2	37	26	0

With regard to the reduction of nitrates, the white and colorless forms show generally negative results. Nitrites are produced by one in 10 of the yellows, a slightly higher fraction of the orange forms, and by half the red-pigment-producers. Ammonia production, on the other hand, appears in one in eight of the yellows, one in 10 of the orange forms, and not at all among the reds.

TABLE 39.
CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	White	Yellow	Orange	Red
20°	4	66	13	0
20° or 37°	28	156	126	25
37°	8	32	42	0

Excluding the majority of forms which grow equally at either temperature, it appears that, among the white and orange forms, most of those which exhibit any preference grow best at 37°, while among the yellows 20° is more often the optimum. These results accord with the habitats, respectively parasitic and saprophytic, of the two classes.

TABLE 40.
CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR COLOR FORMATION.

Color Production	White	Yellow	Orange	Red
Better at 20°	2	90	125	21
Equal at 20° and 37°	38	164	56	4

It appears from Table 40 that temperature differences affect the production of orange pigment much more than that of yellow and that the body temperature interferes with red chromogenesis most of all.

TABLE 41.
CORRELATION BETWEEN CHROMOGENESIS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction, cm.	White	Yellow	Orange	Red
0.0	27	83	55	21
0.1-1.5	8	126	39	4
1.6 and over	5	45	87	0

The liquefaction of gelatin presents another close correlation with pigment production. The white and red forms are almost all non-liquefiers, the yellow cocci show a maximum among the

moderate liquefiers, and the orange chromogens exhibit the peptonizing power to a high degree.

To sum up, the cocci show four (or five) distinct groups according to their pigment production, each group being marked by a number of other correlated characters. The "white" forms rarely show packets, generally stain by Gram, fail to reduce nitrates, grow well at 37°, and usually fail to liquefy gelatin. They include two sub-groups—the feebly growing, strongly acid-producing forms, which are really colorless, not white, and the white-pigment-producers, which grow abundantly and produce only a slight amount of acid. The "yellow" chromogens frequently show packets, are usually Gram-negative, give a good to a very heavy surface growth, produce little or no acid, occasionally form nitrites or ammonia in nitrate solution, grow well at 20°, and show a moderate liquefaction of gelatin. The "orange"-pigment-formers are very rarely packets, stain well by Gram, form good surface growths, produce a moderate acidity in sugar broth, occasionally reduce nitrates to nitrites or ammonia, grow well, but with poor pigment production, at 37°, and generally produce a considerable liquefaction of gelatin. The red-pigment-producers are often packets, generally Gram-negative, grow abundantly, ferment dextrose but not lactose, form nitrites, but not ammonia, in nitrate solution, grow well at 20° or 37°, producing less pigment in the latter case, and generally fail to liquefy gelatin.

9. GELATIN LIQUEFACTION.

In the routine determination of gelatin liquefaction we have used only one tube for each culture. Duplicate determinations were made on 79 cultures, from which it appeared that the probable error of a single observation is only ± 0.12 cm.; so that our method was sufficiently accurate.

Of the cultures observed, 186 failed to liquefy gelatin, and the distribution of the other 314 cultures according to the amount of

TABLE 42.
GELATIN LIQUEFACTION.

Depth in cm.	0.1-0.5	0.6-1	1.1-1.5	1.6-2.0	2.1-2.5	2.6-3.0	3.1-3.5	over 3.5
Number of cultures	33	76	68	48	44	29	13	3

liquefaction after four weeks is shown in Table 42. Fig. 4 shows graphically the skew curve plotted from these data.

There is, as would be expected, a gradual falling-away toward the highest amounts of liquefaction, and the abrupt downward falling of the curve toward the non-liquefiers at 0 is extremely significant as indicating a sharp difference between the two groups. If it had been practicable to plot the non-liquefiers on this figure, the curve would have gone up at an acute angle more than twice as high as that of the mode of the liquefiers. This angle divides with more than usual definiteness those organisms which liquefy from those which do not liquefy gelatin.

The correlations of gelatin liquefaction with other properties have been already considered. We have found that a high peptonizing power is rare among the earth and water cocci and the sarcinæ. It is most frequently associated with the smaller individual cells among the non-packet-formers. It is absent or very rare in the cocci which show only faint surface growths. Finally, it appears that the white, colorless forms which produce high acidities, as well as the red chromogens, are non-liquefiers. The yellow cocci which produce little acid are moderately active liquefiers, and the orange forms with a moderate acid production show the highest peptonizing power.

IV. CONCLUSIONS FROM THE INVESTIGATION.

I. FOUNDATION OF SUBFAMILIES AND GENERA AMONG THE COCCI.

The extreme variability of the cocci has appeared with great clearness in the present study. Almost every one of the characters measured shows a wide range of fluctuations. In view of the general laws of variation, the absence of sexual reproduction, and the susceptibility of the bacteria to the direct influence of the environment, this is precisely what should be expected. It makes it, however, clearly impossible to draw sharp and arbitrary lines for any single character by which individual organisms can be naturally classified.

If, on the other hand, we examine a series of individuals with the idea of discerning central types about which they vary, the problem begins to solve itself, since such types are easily apparent. Certain organisms tend to show the packet grouping—some invariably

in every aggregate, some less constantly. Other organisms never show packets, or only very rarely. Some cocci always stain, and some always decolorize, by Gram, while intermediate forms tend more or less strongly toward either type. In surface growth two distinct types, the faint to meager and the abundant to very heavy, are manifest. In acid production there appear to be three centers of distribution corresponding to organisms which fail to ferment, those which ferment slightly, and those which produce large amounts of acid. In relation to nitrate reduction, three types appear, according as the cocci fail to reduce, or form nitrites or ammonia. On gelatin the organisms studied group themselves either as liquefiers or as non-liquefiers, and in color production four distinct centers appear, in which the pigment is white, yellow, orange, or red.

Our estimate of the value of these type-centers is greatly increased when we find that the central points for the different characters do not vary independently, but are correlated together to a remarkable degree. Again, we should expect, and we actually find, in some cases, the correlation of single characters varying, those properties generally correlated appearing in certain organisms in exceptional combinations. If, however, we consider, not the single character—not the individual organism—but the aggregate of the correlations of various properties as manifested in a considerable series of individuals, certain well-defined systematic units appear, marked by the association of a number of independent characteristics. Such an association can be explained only on the ground of relationship, and the types marked by the simultaneous occurrence of a number of properties may rightly be taken as the centers from which other, more aberrant individuals have varied.

The fact that correlation exists shows that, on the average, the fluctuations of these characters do not occur independently, but are so closely bound up with these of other properties as to vary together with them. This may be because the selective action of the environment produces a parallel change in each, or because the two characters are so closely bound together, in the physiological balance of the organism, that a change in one leads to a corresponding variation in the other. In either event it is clear that the larger systematic units (families or genera) must be marked by these pro-

found modifications of the whole center of gravity of the organism, and the smaller groups by those characters which, though perhaps showing sharper individual differences, vary by themselves without affecting any other properties.

Our object therefore has been, not to establish arbitrary boundary lines, but to discover existing natural types distinguished by the association of independent characters. In such a task it is obvious that those characters are most important which show the most marked correlations. What these characters are must be determined by study in each particular group. Chromogenesis or gelatin liquefaction may be of generic value in one family, or may mark only varieties in another, as it is, or is not, correlated with a number of other properties. In the Coccaceæ, for example, the liquefaction of gelatin and the reduction of nitrates appear, when judged by this standard, to be of less importance than most of the properties studied. In some cases they appear to be significant, but, in most of the groups indicated, liquefying and non-liquefying forms, and reducing and non-reducing forms, run parallel. Distinctions based on such a single character alone may have specific, but certainly not generic, value. On the other hand, we have been somewhat surprised to find that such apparently fluctuating characters as chromogenesis and the reaction to the Gram stain are strongly correlated with a number of other properties.

A general survey of the whole field of variation among the Coccaceæ indicates clearly the existence of two main sets of correlated characters, corresponding to the subfamilies which we have suggested in a previous communication (Winslow and Rogers, 1905). Habitat, morphology, staining reactions, surface growth, acid production, optimum temperature, and chromogenesis, all vary simultaneously in one or the other of two directions, defining the two subfamilies Paracoccaceæ and Metacoccaceæ. The first group, comprising most of the forms from the body, shows, as a rule, chains and irregular cell-grouping, stains by Gram, yields a meager or only fair surface growth, forms acid in carbohydrates, and produces no pigment, or a white or an orange one. The other group, from earth and water for the most part, often shows packets, decolorizes by Gram, grows well on artificial media, fails to ferment carbohydrates, and produces a

yellow or red pigment. It must always be remembered that each character may occasionally be found in the group where it usually does not occur; but the association of these properties in the vast majority of cases is very strong. We desire to extend our earlier definitions of the two subfamilies by including the Gram reaction and chromogenesis; and the subfamilies as thus modified will be defined at the end of this communication. It is a striking fact that these two chief divisions among the Coccaceæ correspond to the two markedly different environments which exist in nature, the body of higher organisms, and the outer world. A close correspondence with environmental conditions should naturally be expected among such simple asexual organisms as the bacteria, and it increases our confidence in the reality of the groups established below to find each of them localized so sharply in one or other of the two main environments.

Under the subfamilies we find a second grade of group-individuality, marked by the association of a smaller number of characters than the subfamilies, but still defined by the correlation of several independent properties. Here morphology, surface growth, and chromogenesis appear to be of greatest importance, acid production, gelatin liquefaction, and nitrate reduction having special significance in certain cases. Five distinct types have appeared with considerable clearness in the present study. It must be remembered that the fundamental correlations which revealed these groups were derived in an entirely impersonal way by measurements, made on each character independently, generally by different observers, and always without knowledge of the identity of the organism. When individual races are considered, it is possible, by transferring a few cultures on the border-line in a single character, to show that the correlations are really closer than have appeared above.

By this process we have attempted to group our 500 cultures under the five subdivisions suggested by the correlation tables, and have found the results so satisfactory as to confirm our confidence in their reality as natural groups. It seems to us that these groups are of such importance as to deserve generic rank. Within each there is ample room for the establishment of such a reasonable number of species as detailed study may warrant. Good genera must first be recognized, however. It is time that bacteriologists

were relieved of such vast and unwieldy and meaningless genera as now burden the science.

The first of these groups centers about a type of organism characterized by the following properties: it is parasitic in habit and grows in irregular groups, often in chains, never in packets; it stains by Gram; it grows in a thin film on the surface of agar; it ferments both lactose and dextrose with the production of a large amount of acid; it fails to reduce nitrates or liquefy gelatin, grows best at 37° and forms no appreciable amount of pigment. This corresponds to the genus *Streptococcus* (Billroth) W. and R., as previously characterized. We desire to add to our previous conception of the group the positive reaction to the Gram stain and the general failure to act on gelatin or nitrates. It must always be remembered that this genus is defined, not on morphology alone, although its members generally do show long chains in broth, but by the general complex of all its characters. Individual cultures vary from the type in some respects, as must all aggregates of organisms composed of such varying stuff as living protoplasm.

Of our 500 cultures 18 fall into the genus *Streptococcus*. All show the typical morphology (groups and long and short chains) and typical surface growth. None liquefy gelatin or reduce nitrates. Of our 18 cultures, 15 were from the body and three from polluted water. In relation to the Gram stain, 11 cultures showed positive tests, on both trials, and only two a negative test, five being variable. Of the 18 cultures, nine showed very high acidities, over 2 per cent normal, in both acids, some ranging as high as 8 to 9 per cent. The average value for the whole genus is, for dextrose 2.6 per cent, and for lactose 1.7 per cent. It is interesting to notice that those cultures which yield lower acidities are also the ones which give the negative or variable Gram reactions. Our forms therefore seem to fall into two species, 10 of them belonging to the *Str. erysipelatos*, showing the very high acidities and the positive Gram reaction; the other eight differing in both these characters.

The second of our five groups is marked by a correlation of characters, of which the most obvious is the production of an orange pigment. In our previous communication we were unable to distinguish, from the literature alone, any sharp line between the orange

and yellow chromogens, and included them both under the genus *Micrococcus*. Fig. 3 makes it clear, however, that two distinct centers of variation exist, one in the orange and one in the yellow, and our correlation tables show that the two types of organisms are so radically different in every character as to demand their separation into distinct genera. Furthermore, it is evident that the orange chromogens belong with the parasitic Paracoccaceæ, and the yellow forms with the Metacoccaceæ. Nothing could show more clearly how necessary it is to make a comparative study of a large series of organisms in order to discern the true relationships of the bacteria.

For this new genus we suggest the name *Aurococcus*, as indicating the orange color, which is its most obvious characteristic. Its type-form is found on or in the plant or animal body. It occurs in groups and short chains, stains by Gram, and produces a good, but not heavy, surface growth of an orange color. It ferments dextrose and lactose, producing an acidity generally between 0.5 and 1. It grows well, but produces less pigment at 37°. It may or may not reduce nitrates and liquefy gelatin. When it does liquefy gelatin, it does so rather actively.

Of the 158 cultures in this group, all show a good, but not very abundant, growth of an orange color; 116 were obtained from the body and 30 from the air, only 12 having a saprophytic origin; 147 show groups and short chains, but no packets; and 11 occasionally give the sarcina grouping. Of the 158 cultures, 107 show a positive Gram reaction, and only nine a consistently negative one. The average acidity in dextrose for the whole group is 0.7 per cent normal, and for lactose 0.4 per cent normal. Of the 158 cultures only six form less than 0.2 per cent acid, and 17 more than 1 per cent acid, in dextrose. In lactose there is more variation; 53 cultures give less than 0.2 per cent, and 11 more than 1 per cent, acid. Of the cultures, 31 reduce nitrates, and 102 liquefy gelatin to an average depth of 2.2 cm.—a very high value; while 56 organisms fail to liquefy. The type-form of this genus is the commonest pyogenic organism, the *M. aureus* of Rosenbach. The non-liquefying forms, those which reduce nitrates, and those which produce more or less acid than is common in the genus, may later be set up as separate species.

The third of our types, like the second, has not previously been distinguished from the genus *Micrococcus*; it appears, however, to show its own definite individuality, and to belong with the Paracoccaceæ, although it approaches the saprophytic cocci in certain characters. We suggest the name *Albococcus* for this genus, which includes those organisms of which *M. pyogenes* (Ros.) Mig. is a type. They produce a more vigorous surface growth than the streptococci, with a clear white pigment, and ferment carbohydrates, producing a fair amount of acid. They are also distinguished from the Metacoccaceæ by the general tendency of their morphology and staining reactions, and by their habitat. In our series we have 23 cultures of this type. All without exception were obtained from the body or from the air, none from water or earth. All without exception show a good surface growth, white pigment, and division into groups and rarely chains, but never packets. Sixteen were uniformly Gram positive and only two uniformly Gram negative. The average acidity in dextrose broth was 0.7 per cent normal, and in lactose broth 0.5 per cent normal. Only three cultures showed an acidity lower than 0.2 per cent, and only one culture an acidity over 1.5 per cent in dextrose. Lactose results, as usual, were more variable, nine cultures falling below 0.2 per cent acid, and one above 1.5 per cent. Nitrates were reduced by three cultures and gelatin liquefied by 14. The four species which we have previously called *M. pyogenes* (Ros.) Mig., *M. rhenanus* Mig., *M. candicans* Flüge, and *M. canescens* Mig., should belong to this new genus, being distinguished, as before, by their relation to acid and gelatin. The reduction of nitrates may furnish a basis for the establishment of other species.

The fourth of the general groups which have appeared in this study is the group of the yellow pigment formers, of which *M. luteus* and *Sarcina ventriculi* are typical—a group which differs in almost all its properties from those previously considered. Organisms of this type are found mainly in earth and water rather than on or in the animal body. They give abundant, to very heavy, growths of a yellow color. They frequently occur in packets, generally decolorize by Gram, and fail to ferment sugars or ferment them only slightly. They may or may not liquefy gelatin or reduce nitrates.

Of our cultures 262 fall under this head, 195 of them coming from water, earth, or air, and only 64 from the body; 200 are uniformly or at times Gram negative, and only 62 uniformly Gram positive. The average acidity produced in dextrose broth is only 0.2 per cent normal, and in lactose broth 0.1 per cent normal. Of the 262 cultures only 33 give over 0.5 per cent acid in dextrose broth, and only 7 over 0.5 per cent acid in lactose broth; 59 of the cultures reduce nitrates; 85 fail to act on gelatin, and 177 liquefy it, producing an average liquefaction of 1.2 cm., scarcely more than half the value in the genus *Aurococcus*.

This group divides itself, according to cell-grouping, into two nearly equal divisions—those which form packets and those which do not; 136 belong to the former class, and 126 to the latter. In habitat, in Gram staining, and in relation to carbohydrates and gelatin both classes are entirely parallel. The genera *Micrococcus* and *Sarcina* are, however, so firmly established in common usage that it would require very strong evidence of identity to warrant dropping either of them. It seems best, therefore, to recognize the single character of cell-grouping as having generic value in this case, otherwise defining the two genera by the same characteristics. Under *Micrococcus* will come *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal; under *Sarcina*, *St. subflava* Ravenel and *S. ventriculi* Goodsir.

The fifth and last of our general types includes the sharply marked one of the red chromogens. These are entirely saprophytic forms which produce abundant surface growths of a red color. They may or may not show packets, are generally Gram negative, very rarely liquefy gelatin or ferment carbohydrates, and frequently reduce nitrates to nitrites, but apparently not to ammonia. This is the first case in which we have found the action upon nitrates markedly correlated with other characters.

Twenty-five of our cultures fall under this general type. All but one come from earth, water, or air. Only three are uniformly positive to Gram, while 15 are uniformly negative. Four of the 25 cultures liquefy gelatin, and 14 reduce nitrates to nitrites. The average acidity in dextrose broth is 0.4 per cent normal, and in lactose the average reaction is neutral. One culture in lactose and four in dextrose show an acidity over 0.5 per cent.

Here again the packets (10 in number) and the other forms (15 in number) are exactly parallel. Both show the same range of acidities and the same peculiar relation to nitrate reduction. It seems quite clear that in this case the single character of packet formation ought not to be made the basis of a distinct genus. We have recognized among the yellow forms the two genera *Micrococcus* and *Sarcina* out of deference to custom, which must always play an important part in terminology. In separating the red forms from these two old genera, however, it seems an unreasonable recognition of a false distinction to form two new ones on the single character of cell-grouping alone. We desire, therefore, to include all the red-pigment forms, characterized by the properties noted above, under one new genus, to be called *Rhodococcus*. *M. cinabareus* Flügge, *S. rosacea* Lindner, and *S. incarnata* Gruber will all belong here. Fourteen doubtful cultures are for the present omitted from this generic classification.

It remains only to summarize the characteristics of the six genera studied in this investigation in tabular form, and then to present a systematic statement of the main divisions of the Coccaceæ. It must be remembered that *Diplococcus* and *Ascococcus* have not been included in the present research and are defined solely from the literature.

TABLE 43.
CHARACTERS OF CERTAIN GENERA OF THE COCCACEÆ.

Genus	Habitat (Per Cent Parasitic Forms*)	Cell-Grouping (Per Cent of Packet-Formers)	Gram-Stain (Per Cent of + Results)	Surface Growth	Average Acidity in Dextrose (Per Cent Normal)	Average Acidity in Lactose (Per Cent Normal)	Nitrate Reduction (Per Cent of Reducers)	Chromogenesis	Gelatin (Per Cent of Liquefiers)	Liquefaction (Average Liquefaction*, cm.)
<i>Streptococcus</i>	83	0	61	Faint.....	2.6	1.7	0		0	...
<i>Aurococcus</i>	76	7	68	Good.....	0.7	0.4	21	Orange.	65	2.2
<i>Albococcus</i>	48	0	70	Abundant....	0.7	0.5	13	White..	61	1.1
<i>Micrococcus</i>	27	0	25	Good to Abun.	0.3	0.1	27	Yellow.	68	1.2
<i>Sarcina</i>	22	100	23	Good to Abun.	0.2	0.1	18	Yellow.	67	1.2
<i>Rhodococcus</i>	4	40	12	Abundant....	0.4	0.0	56	Red...	16	0.7

* Body alone; air, source of many others. In *Albococcus* none from water or earth.

2. SYSTEMATIC SUMMARY.

Family Coccaceæ: Vegetative cells spherical.

Subfamily 1 Paracoccaceæ (Winslow and Rogers): Parasites (thriving only or best, on or in, the plant and animal body). Thrive

well under anaërobic conditions. Many forms fail to grow on artificial media; none produce very abundant surface growths. Planes of fission often parallel, producing pairs, or short or long chains, never packets. Generally stain by Gram. Produce acid in dextrose and lactose broth. Pigment, if any, white or orange.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule. Includes *D. pneumoniae* Weich., *D. Weichselbaumii* Trev., and *D. gonorrhoeae* Neisser.

Genus 2, *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. On agar streak effused, translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates. Includes *S. erysipelatos* Fehleisen.

Genus 3, *Aurococcus*, new genus: Parasites (see above). Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth of orange color. Sugars fermented with formation of small amount of acid. Gelatin often liquefied, very actively. May or may not reduce nitrates. Includes *A. aureus* (Rosenbach).

Genus 4, *Albococcus*, new genus: Parasites (see above). Cells in groups and short chains (never in packets). Generally stain by Gram. Growth on agar streak abundant and porcelain white in color. Sugars fermented with production of a slight amount of acid. Gelatin liquefaction and nitrate reduction may or may not occur. Includes *A. pyogenes* (Rosenbach), *A. rhenanus* (Migula), *A. candicans* (Flügge), and *A. canescens* (Migula).

Subfamily 2, Metacoccaceæ (W. and R.): Facultative parasites or saprophytes. Thrive best under aërobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets, or zoöglea masses. Generally decolorize by Gram. Pigment, yellow or red.

Genus 5, *Micrococcus* (Hallier): Facultative parasites or sapro-

phytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant with formation of yellow pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced. Includes *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal.

Genus 6, *Sarcina* (Goodsir): Exactly like *Micrococcus*, except that division occurs under favorable conditions, in three planes, producing regular packets. Includes *S. ventriculi* Goodsir, *S. aurantiaca* Flügge, *S. subflava* Ravenel, *S. tetragena* (Mendoza) Mig.

Genus 7, *Rhodococcus*, new genus: Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant, with formation of red pigment. Dextrose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced to nitrites, but not to ammonia. Includes *R. cinnabareus*, Flügge, *R. roseus* Flügge, *R. fulvus* Cohn, *R. agilis* (Ali Cohen), *R. rosaceus* Lindner, and *R. incarnatus* Gruber.

V. REFERENCES.

- ARONSON, H., 1903. *Deutsche med. Wchnschr.*, 29, p. 439.
 BIGELOW, R. P., 1904. *Wood's Reference Handbook of the Medical Sciences*, 8, p. 182.
 BUERGER, L., 1904. *Med. News*, 85, p. 1117.
 CHESTER, F. D., 1901. *A Manual of Determinative Bacteriology*, New York.
 CHESTER, F. D., 1904. *Fifteenth Annual Report of the Delaware College Agricultural Experiment Station for 1903*.
 CLARK, H. W., AND GAGE, S. D., 1905. *Eng. News*, 53, p. 27.
 COMMITTEE ON STANDARD METHODS OF WATER ANALYSIS, 1905. *Jour. Infect. Dis.*, Supplement No. 1, p. 1.
 CONN, H. W., 1900. *Jour. Boston Soc. Med. Sciences*, 4, p. 170.
 DUNHAM, E. K., 1903. *Abstr., Science*, N. S., 17, p. 372.
 ELLIS, D., 1902. *Centralbl. f. Bakt.*, Abt. I, Orig., 33, p. 1.
 FISCHER, H., 1904. *Centralbl. f. Bakt.*, Abt. I, Orig., 37, p. 449.
 FLÜGGE, 1896. *Die Mikroorganismen*, Leipzig.
 FULLER, G. W., AND JOHNSON, G. A., 1899. *Jour. Exper. Med.*, 4, p. 609.
 GAGE, S. D., AND PHELPS, E. B., 1903. *Rep. and Papers Amer. Public Health Assoc.*, 28, 1902 meeting, p. 494.
 GALTON, F., 1889. (a) *Proc. Roy. Soc.*, 45, p. 136; (b) *Natural Inheritance*, London.
 HOWE, F., 1904. *Centralbl. f. Bakt.*, Abt. I, Orig. 36, p. 484.
 JORDAN, E. O., 1903. *Jour. Hyg.*, 3, p. 1.
 KENDALL, A. I., 1903. *Rep. and Papers Amer. Public Health Assoc.*, 28, 1902 meeting, p. 481.
 KERNER, J., 1905. *Centralbl. f. Bakt.*, Abt. I, Orig. 38, p. 223.

- KOLLE, W., AND OTTO, R., 1902. *Ztschr. f. Hyg.*, 41, p. 369.
- MEYER, A., 1903. *Practicum der botanischen Bakterienkunde*, Jena.
- MEYER, F., 1902. *Berl. klin. Wchnschr.*, 39, p. 936.
- MIGULA, W., 1900. *System der Bakterien*, Band II, Jena.
- NEUMANN, R., 1897. *Archiv f. Hyg.*, 30, p. 1.
- OTTO, R., 1903. *Centralbl. f. Bakt.*, Abt. I, Orig. 34, p. 44.
- PEARSON, K., 1900. *The Grammar of Science*, London.
- QUETELET, A., 1846. *Lettres . . . sur la théorie des probabilités, appliquée aux sciences morales et politiques*, Brussels.
- RIPLEY, W. Z., 1899. *The Races of Europe*, New York.
- ROBINSON, B. L., 1906. *Science*, N. S., 23, p. 81.
- SMITH, T., 1900. *Jour. Boston Soc. Med. Sciences*, 4, p. 95.
- SMITH, E. F., 1905. *Bacteria in Their Relation to Plant Diseases*, I, Carnegie Institute, Washington.
- SULLIVAN, M. X., 1905. *Jour. Med. Research*, 14, p. 109.
- THORNDIKE, E. L., 1904. *Introduction to the Theory of Mental and Social Measurements*, New York.
- WESTON, R. S., AND KENDALL, A. I., 1902. *Rep. and Papers Amer. Public Health Assoc.*, 27, 1901 meeting, p. 402.
- WHIPPLE, G. C., 1902. *Technol. Quarterly*, 15, p. 127.
- WINSLOW, C.-E. A., AND ROGERS, ANNE F., 1905. *Science*, N. S., 21, p. 669.
- WOODS, F. A., 1906. *Mental and Moral Heredity in Royalty*, New York.

THE TOXIC EFFECT OF CERTAIN ACIDS UPON TYPHOID AND COLON BACILLI IN RELATION TO THE DEGREE OF THEIR DISSOCIATION.*

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I. INTRODUCTION.

THE researches of the physical chemists, under the leadership of Arrhenius and Nernst, have shown that certain substances in aqueous solution become dissociated or broken up into electrically charged part-molecules (atoms or groups of atoms), which are called ions. The extent to which this occurs varies with different substances and is greatest in the most dilute solutions. With strong acids and bases, and their salts, it is practically complete at a strength of 0.001 normal. With such solutions it is evident that any effect, chemical or physiological, which they exert, must be due to the dissociated ions. The properties of a dilute solution of sodium chloride are the properties of sodium and chlorine ions, and the properties of hydrochloric acid, of hydrogen and chlorine ions. By the comparison of a series of properly selected compounds it is easy to determine the specific influence of each ion. The study of the toxic action of various substances in the light of these facts promises to be of great assistance in the development of a rational theory of disinfection.

The first definite statement of the relation between dissociation and disinfectant power with which we are familiar was made by Dreser. This author (Dreser, 1893), in a study of the pharmacological value of various salts of mercury, found that the double hyposulphite of mercury and potassium was much less poisonous than other compounds containing the same amount of mercury, and explained the phenomenon by the fact that this salt on dissociation does not set free mercury ions, but breaks up into potassium at the cathode and $\text{Hg S}_4\text{O}_6$ at the anode. His experiments were made on yeast cells, frogs, and fishes. In the former case he found it possible to prevent all development in a yeast culture by mercury salts, and

*Received for publication March 5, 1906.

then by the addition of potassium hyposulphite to permit fermentation without precipitating any of the mercury, simply by the formation of the differently dissociated double salt.

Scheurlen and Spiro (1897) confirmed the conclusions of Dreser as to the correlation between dissociation and disinfectant action among the mercury salts, and extended them to cover certain compounds of iron. At the same time they maintained that in other cases (the ethylchlorid and ethylsulphate of mercury) strong disinfectant action was apparently due, not to free ions, but to the undissociated molecule.

A number of phenomena which had long been empirically familiar in bacteriology found an easy explanation on the electrolytic theory of disinfection. The effect of temperature in increasing the activity of disinfectants, for example, had been pointed out by Koch (1881), and later by Behring (1890) and Heider (1892), and many others. It was at once obvious that this might be due in some cases to the increased dissociation at high temperatures. It would be well worth while today to see how far the increased activity of disinfectant runs quantitatively parallel to its dissociation. Again, Minervini (1898), and other investigators, have shown that various antiseptic agents (carbolic acid, chromic acid, mercuric chloride, and silver nitrate in Minervini's experiments) are much less active in alcoholic than in aqueous solutions. This fact, too, is easily explicable as due to the diminished dissociation in such solvents.

The relation between dissociation and toxicity was put upon a sound quantitative basis by the work of Krönig and Paul, first published in 1896 (Paul and Krönig, 1896), and in fuller detail in the next year (Krönig and Paul, 1897). These authors carried out an elaborate series of experiments on the disinfectant action of various salts, bases, and acids in the light of the new conclusions of physical chemistry. The details of the investigation were arranged with the greatest care in order to secure comparable results. Spores of *Bacillus anthracis* and vegetative cells of *Micrococcus aureus* were used, dried on Bohemian garnets. By using a definite number of garnets of a certain size shaken up with a suspension of an agar culture, after filtering through paper, and carefully drying, it was found possible to expose approximately the same number of cells in each experiment.

The garnets were dried for 12 hours at 7° and exposed in platinum sieves to the action of the disinfectant solution studied. The temperature was kept constant at 18° C. during the experiment, and after the desired time had elapsed the excess of disinfectant was carefully removed by appropriate reagents (neutralization of acids and bases, precipitation of heavy metals with ammonium sulphide, etc.). After thorough rinsing, the garnets were shaken up with water to detach the cells, which were then plated on agar. No attempt was made accurately to fix a killing point by testing a long series of dilutions of each disinfectant, and no exact calculations were made of dissociation. In a general way, however, the number of spores of *B. anthracis* which developed after treatment for a given time varied inversely with the amount of dissociation. Thus in the study of metallic salts it appeared that the activity of various compounds of mercury, silver, copper, and gold was greatest in those actively dissociated, and decreased in those which yield less free metallic ions. Solutions of mercuric chloride and silver nitrate, in alcohol, where no dissociation occurs, showed almost no disinfectant action. Furthermore, the toxic action of a salt having poisonous metallic ions was markedly diminished by the presence of a non-toxic salt of the same acid. This is in accord with physico-chemical theory; in any solution the ratio between the undissociated molecules and the product of free anions and cations is constant, so that the addition of sodium chloride to mercuric chloride keeps the proportion of chlorine ions the same, but replaces a portion of the mercury ions by those of sodium. So it appeared in Krönig and Paul's work that successive additions of sodium chloride to mercuric chloride progressively increased the number of colonies developing after the usual treatment. In the study of different salts of the same metal it was found that the acid radical may also exercise considerable influence on the disinfecting power.

With bases Krönig and Paul found the same general relation to hold, ammonium hydroxid, which is weakly dissociated, being a much less active disinfectant than the corresponding compounds of potassium, sodium, and lithium. The authors noted in a general way a diminution of disinfectant action in the presence of organic compounds. The decrease was most marked with the halogens and

oxidizing agents, and less with acids and bases. Disinfectants themselves of organic nature were least affected.

The particular phase of the subject with which we are especially concerned, the disinfectant action of the acids, was not exhaustively treated in this investigation. One series of experiments was made with normal and half-normal solutions, in which it was found that hydrofluoric, nitric, and trichloroacetic acids in normal strength killed all the anthrax spores in 120 minutes. Normal chloric, hydrobromic, and hydrochloric acids and half-normal oxalic acid left a few spores alive after eight hours. Normal sulphuric acid was a little less effective, and normal phosphoric, formic, and acetic acids left large numbers of organisms alive after eight hours. Hydrocyanic acid in normal strength showed little action even after 30 hours. The investigators conclude that there is a general relation between the action of the acids and the amount of dissociated hydrogen present; but there appear many exceptions to a strict parallelism. The authors attribute these exceptional effects to the anion or the undissociated molecule, and point out that in more dilute solutions they tend to disappear. Thus, 0.06 normal solutions of hydrochloric, chloric, nitric, and trichloroacetic acids showed about the same disinfectant action, apparently due to the presence of an approximately equal amount of dissociated hydrogen.

At a still earlier period a somewhat similar series of investigations to those of Paul and Krönig had been carried out in another field. This was a study of the relation between toxicity and dissociation as measured by the effect of various salts and bases, and a long series of organic and inorganic acids, on the higher plants, by Kahlenberg and True (1896). Their method consisted in determining the maximum strength of solution in which seedlings of *Lupinus albus* could grow. The seedlings were exposed for 15 to 24 hours, and their condition determined by their general appearance and by the growth which had taken place. These plants proved very sensitive to the action of dilute acid, a strength of from 0.00008 to 0.00064 normal killing them in almost every case. It is interesting to note that boric acid was endured in 10 times this strength. In general, the poisonous action ran parallel with the degree of dissociation, but certain of the organic acids showed relations of their

own. The authors concluded that "in the case of plants the toxic action of solutions of electrolytes, when dissociation is practically complete, is due to the action of the ions present. When dissociation is not complete, the undissociated part of the electrolyte may also exert a toxic effect." Heald (1896) extended the work of Kahlenberg and True to the seedlings of three other flowering plants, and reached the same general conclusions. All these authors pointed out clearly that the effect of the common mineral acids is due to hydrogen alone, since their anions have almost no strong toxic action when neutral salts are used.

The next work along these general lines was carried out in another field of botany by Stevens (1898), at the University of Chicago, the measure of viability used being the germination of fungus spores. A study had been made by Wüthrich (1892), at a much earlier date on the toxicity of metallic salts and acids for the spores of fungi; and Maillard (1898 and 1899) at about the same time reported experiments on the inhibition of the growth of *Penicillium* by copper salts. In Stevens' experiments the spores were inoculated into hanging-drop preparations of the solutions tested, and examined for development after 24 hours. The five organisms used exhibited marked differences in their susceptibility, although all were much less affected than the phanerogamous seedlings, requiring a strength of 0.01-0.02 normal acid to inhibit germination. The relative toxic effect of various substances was not unlike that observed by Heald and Kahlenberg and True. Mercuric chlorid and various copper salts proved most fatal, the acids and cyanides being less active. By the comparison of various substances it appeared that of the anions, CN, CrO₄, Cr₂O₇, and OH are poisonous, and of the cations, Hg, Cu, and H, while the halogens and SO₄ in dilute solutions exert no influence.

A still more exhaustive study of the effect of toxic agents upon the fungi was made by Clark in the next year (Clark, 1899). This investigator followed the same general method as that of Stevens, exposing spores in hanging-drop cultures to the activity of the agents to be tested. The cultures were divided into four classes: those which grew normally, those which showed irregular or retarded growth, those which failed to develop in the medium tested, but grew after

transfer to fresh beef infusion, and those which entirely succumbed to the action of the toxic substances. The wide difference between the concentration of acid producing, respectively, injury, inhibition, and death was one of the most interesting results of these experiments. As in Stevens' work, it was apparent that the fungi are extremely resistant to disinfectants, and it was necessary to use somewhat concentrated solutions, from 0.008 to 0.287 normal acid, for killing. It is perhaps partly for this reason that, as the author says, "in this study no new evidence has been adduced supporting the theory that the chemical activities of a substance are due wholly or chiefly to the ionized portion." On the other hand, it was held that "in the case of several acids, ionization lessens the chemical activities toward the substances involved in the life-processes of the plant." This conclusion is based on calculations of the specific toxicity of each ion and molecule, obtained by comparing the effects of different compounds varying from each other in one element or group. Thus, hydrochloric acid in the solutions used was over 90 per cent dissociated and since experiments with the similarly dissociated chloride of potassium showed this salt to be practically non-toxic, it is evident that its action was due to the combined effect of the hydrogen ion and the undissociated molecule. Nitric acid, dissociated in the same proportion was much more toxic. Since hydrogen ions are equal in the two cases and since the NO_3 ion is harmless, as shown by experiments with neutral salts, the increased effect must be due to the undissociated part of the molecule of nitric acid. Clark calculates that the toxic value of one molecule of undissociated HNO_3 is 7.7 times that of an ion of hydrogen, so that the acid actually loses nearly seven-eighths of its disinfectant power by becoming ionized.

The effect of sulphuric acid was about the same as that of hydrochloric; since it is less dissociated, the author attributes an appreciable influence to the anion, HSO_4 . Acetic acid, at the strength used, is only 2 per cent dissociated, so that its high toxic effect is due to the un-ionized molecule.

The results obtained in this series of experiments with hydrocyanic acid are also interesting. Krönig and Paul (1897) found this acid almost without effect on anthrax spores, while Kahlenberg and True (1896), on the other hand, record very strong toxic action

on the seedlings of higher plants. In Clark's experiments it proved far more fatal than any other acid, being 70 times as active as hydrochloric. The molecule at the concentrations used is probably only slightly dissociated.

In some ways the most important work upon this subject was the very careful study made by Bial, of the antiseptic action of the hydrogen ion of dilute acids upon yeast. He first became interested in the problem from a consideration of the causes which allow production of gas in the stomach, and carried out his earliest experiments by observing the gas formation in yeast cultures in the presence of various substances present in the normal gastric juice. This series of studies (Bial, 1897) showed that the presence of albuminoid substances or of sodium chloride effected a marked restriction of the antiseptic action of hydrochloric acid. Bial at this time did not apply physico-chemical theories to the explanation of these phenomena; but in another contribution he made a fuller study of the problem. His later experiments (Bial, 1902) were again made with yeast cells, cultivated in fermentation tubes filled with grape-sugar solution to which various amounts of acid had been added; the antiseptic action was inversely registered by the amount of gas produced. The advantage of this method is its great delicacy; the fermentative power of the yeast responds to such extremely minute quantities of acid that the ionic effects are not complicated by other actions which appear in stronger solutions. Bial did not make exact calculations of the amount of dissociated hydrogen necessary to inhibit the yeast, but he found that a general relation existed between the ionization and the antiseptic action. The strongly dissociated acids—hydrochloric, sulphuric, nitric, and trichloroacetic—entirely stopped the action of the yeast in concentrations of between 0.005 and 0.008 normal. Acids of an intermediate grade—phosphoric, formic, and oxalic—accomplished the same effect when 0.01 normal; while acids still less dissociated—acetic, benzoic, and butyric—stopped all fermentation only when 0.04 to 0.07 normal. The most striking feature of Bial's work was a series of experiments on the diminution of the antiseptic action of acids by the addition of neutral salts whose action is to decrease the dissociation of the acidic hydrogen. A solution of 0.01 normal formic acid and 0.3

normal sodium formate showed active fermentation, as did a solution of 0.0166 normal hydrochloric acid and 0.2 normal sodium chloride. The same phenomenon was observed with oxalates, nitrates, sulphates, and acetates. An exhaustive study in the case of hydrochloric acid showed that, while a certain amount of sodium chloride diminished the toxicity of the acid, a much larger amount actually increased it. Bial attributes this to a catalytic action of chlorine ions, but it seems to us that the facts may be explained more simply by the direct inhibiting effect of the sodium chloride and its ions. Bial found that twice normal sodium chloride without any acid prevented fermentation; and it is quite possible, in dealing with living organisms, that the combined effect of the acid and the chloride would be inhibitory at concentrations which with either acid or base alone might allow fermentation to go on. Bial studied also the effect of hydrochloric acid and sodium chloride in the presence of peptone, and found that the yeast would bear more of the salt than in the presence of acid alone.

The experiments of Paul and Krönig demonstrated clearly that in certain solutions disinfectant action runs parallel with the presence of dissociated ions. The work upon the higher plants and the mold fungi confirmed these results, but showed that in other cases the undissociated molecule is of great importance. Bial's studies brought out clearly the influence of neutral bodies, inorganic salts or proteids, in diminishing disinfectant action by decreasing dissociation.

The problem is, of course, complicated by still other chemical interactions which are more obscure. For example, Scheurlen, (1895), Beckmann (1896), Römer (1898), and Spiro and Bruns (1898) have shown that in the case of phenol and certain other organic disinfectants the addition of sodium chloride greatly increases toxic action. Still another factor which affects disinfectant power has been brought out in recent years by Nägeli (1893), and other observers—the presence of suspended solid particles of neutral character. In the most recent communication upon this subject by True and Oglevee (1905) it was shown that the toxic effect of metallic salts upon *Lupinus* may be entirely counteracted by the presence of finely divided particles of sand, glass, filter paper, coal, starch, or paraffin. On the other hand, the toxic effect of organic disinfectants

—phenol, thymol, and resorcinol—was affected only to a barely appreciable degree. The action when it occurs is explained by the power of suspended particles to remove dissolved substances by a process of adsorption, and the possibility suggests itself that the removal of ions by large organic molecules in true or colloidal solutions may be of an analogous character. Whatever the cause, this phenomenon must prove of far-reaching importance in bacteriology. Such facts as the observed multiplication of bacteria, when water samples are stored in glass bottles, may be the result of a removal of inhibiting substances by the adsorptive action of glass surfaces.

A considerable body of evidence in the field of animal physiology bears out these conclusions obtained from the study of bacteria and other plants. A fairly full summary of this literature may be found in the reviews of Cohen (1903) and Hamburger (1904). The work of Kahlenberg (1898) and other observers, who have shown that the taste of dilute solutions is in many cases due to the specific properties of the dissociated ions, is of interest. Studies which have been frequently cited were made by Loeb (1897 and 1898), and recently reprinted (Loeb, 1905), on the influence of free ions upon frog's muscle. The gastrocnemius muscle absorbs water and increases its weight in the presence of slight traces of acids or alkalis, and Loeb concluded that for the inorganic acids and bases this increase in weight is solely a function of the number of hydrogen and hydroxyl ions in the unit of volume. This sweeping conclusion is hardly borne out by his experiments. With the organic acids there was no relation whatever. Trichloroacetic acid, almost entirely dissociated, and lactic acid, with only 11 per cent dissociation, gave practically the same results. With a series of 11 different organic acids of every degree of dissociation, the individual variation in weight-increase, with 0.009 normal solutions, ranged only between 3.9 per cent and 7.2 per cent.

A very significant line of physiological investigation concerns the binding of free ions by organic molecules of large size. In one of the most recent communications on this subject, Stiles and Beers (1905) have shown that the effect of calcium chloride, barium potassium chloride, and sodium nitrite upon plain, cardiac, and striped

muscle of the frog, terrapin, and guinea-pig was reduced from one-half to three-fourths in the presence of white of egg, partially dialyzed serum, peptone, or starch. In some cases a combination between the inorganic body and the proteid has been demonstrated by freezing-point determinations, but in other cases particularly with the neutral salts, this has not been shown. As the authors suggest, these experiments point to the existence of "*physiological compounds* which are not demonstrable at all by chemical methods, but only by the reactions of living tissues."

2. OBJECT AND METHODS OF THE PRESENT INVESTIGATION.

The present investigation was begun with the intention of determining the effect of acid wastes in sewage upon the viability of the typhoid bacillus under practical conditions. It soon appeared, however, that the problem was too complex to be attacked in any general way without the preliminary determination of certain of the individual factors involved, under definitely controlled conditions. We have therefore attempted to find the disinfectant power of two mineral acids and two organic acids upon the typhoid bacillus in tap water and in the presence of peptone, and have controlled these experiments by a parallel series with the colon bacillus. The results, besides their specific value as determinations of the reactions of these two organisms to dilute acids, have a certain interest in relation to the general theory of disinfection. In all the experiments reviewed above, except Bial's, the acids used were tested in only a few widely differing strengths, so that the parallelism between disinfectant action and dissociation was not established with any great exactness. In the work of Krönig and Paul on anthrax spores and the various studies on the mold fungi, it was necessary to use such strong solutions that ionic effects were largely masked by the influence of the undissociated molecule, and in the studies of Kahlenberg and True and Heald on the phanerogams it was evident that with such complex organisms many other factors than the direct effect on protoplasm come into play. There was room, therefore, for a series of experiments on organisms sensitive to very dilute acids, carried out in sufficient detail to show definitely the relations between toxicity and dissociation.

With the view of securing exact quantitative results, we adopted the method of exposure to the acid tested, in a suspension from which samples were directly plated. This process has the obvious defect of permitting a certain amount of the disinfectant to be carried over to the plate, where it may exert an antiseptic action. We have really measured the combined disinfectant action in the suspension and possible antiseptic action in the plate. The action of organic matter in decreasing toxicity, shown by our experiments, must greatly reduce any such action in the plate.

The procedure in each experiment was as follows: A series of bottles, each containing 100 c.c. of sterile water (or peptone solution), was arranged in a row, and to each bottle was added a different amount of standardized acid from a graduated pipette. The amount of water in each bottle was measured at the end of the experiment, in order to obtain the exact strength of the solution. Immediately after the addition of the acid there was added to each bottle 1 c.c. of a fresh aqueous suspension of the bacteria tested. After standing for 40 minutes, lactose agar plates were made in duplicate, from the acidified bottles, and from controls with no acid. Colonies were counted after 24 hours' incubation at 37° C.

Forty minutes was selected, after some preliminary experiments, as the best period of exposure to the acid, since it gave sharper results than a shorter time. In the tests reported in the accompanying tables there was not a variation from the 40 minutes of more than one minute in most of the samples. The series for *B. typhi* in water, with HCl, were also examined after 100 minutes, and after 24 hours. In the sample containing 48 parts per million of sulphuric acid, there was, after 40 minutes, 59.3 per cent removal of *B. typhi*; after 100 minutes, 88.15 per cent removal; after 24 hours, 100 per cent removal. The sample containing 92.9 parts per million of sulphuric acid removed after 40 minutes 92.97 per cent; after 100 minutes, 99.99+ per cent; after 24 hours, 100 per cent. The removal was 100 per cent in all of the samples containing larger amounts of acid after 103 minutes, and in all of the samples after 24 hours.

The temperature factor was of considerable importance, and care was taken to keep conditions uniform. No agar was poured with a temperature greater than 50° C. It was found that a rise in tem-

perature in the presence of the acid was very fatal in its effect on the bacteria.

The typhoid culture used was obtained from the Massachusetts General Hospital, where it had been isolated from the spleen of a clinically typical case of typhoid fever; the colon bacillus was isolated in the laboratories of the Institute, and both gave all characteristic reactions. Twenty-four-hour agar-slant cultures were used in all cases.

The tables have been prepared to show, in the first column, the parts per million of the acid, and in the second column the parts per million of acidic hydrogen or, more accurately, of replaceable hydrogen. The third column shows the strength converted into terms of normality. The percentage dissociation of the acids at each dilution is given in the fourth column, and the actual parts of dissociated hydrogen in the fifth. The last two columns show the initial number of bacteria used as shown by blank controls and the percentage reduction after 40 minutes.

The tables show in general that with increasing quantities of disinfectant the bacterial reduction proceeds rapidly up to a certain point. After 99 per cent of the organisms have been killed, however, it takes a very considerable further increase of acid to produce sterilization. This is a point of very fundamental importance, and one which has been observed in studying the effect of such various agents upon the bacteria, that it deserves special attention. Sedgwick and one of us (Sedgwick and Winslow, 1902) have called attention to the persistence of a few specially resistant individuals when typhoid bacilli are exposed to the action of cold. After 14 days of exposure to freezing temperature 99.8 per cent of the organisms were killed, but after three months a few still survived. Johnson's tables of the reduction of typhoid and colon bacilli by copper salts (Johnson, 1905) show the same phenomenon, although he does not comment upon it specifically. More recently, Frost and Swenson (1906), and Gage and Stoughton (1906), have emphasized this peculiar phenomenon, in connection with resistance to high temperatures. The former authors, working with *B. dysenteriae*, found that "the majority of the cells were killed between 55° and 60°, but that frequently a relatively small number, possibly one individual

in a hundred thousand or a million, may persist at much higher temperatures, even 70° ." Gage and Stoughton in their conclusions point out that "the great majority of the bacteria in any *B. coli* cultures are destroyed by five minutes' exposure to some temperature between 50° and 60° C. A few individuals, however, in each culture will survive much higher temperatures, in some cases remaining alive after exposure to 90° C. The very close range (about 10° C.) of temperature at which the destruction of the majority of the individual bacteria occurred, as compared with the considerable range (about 35° C.), in the temperatures at which complete sterilization was effected would indicate that the determination of the majority death-point would be of more value in species identification than is the determination of the absolute thermal death-point as at present employed."

Altogether it seems clear that among what are ordinarily considered non-sporing bacteria there exists a small proportion of individuals having specially high resistant powers against unfavorable conditions. The absolute death-point for these resistant forms is difficult to determine accurately on account of their small numbers and the consequent chances that they may be overlooked. We are inclined from our experience to agree with Gage and Stoughton as to the superior value for many purposes of the majority death-point (99 per cent), and we shall lay special stress on this in interpreting our results.

3. THE DISINFECTANT ACTION OF HYDROCHLORIC ACID AND SULPHURIC ACID UPON *B. TYPHI* AND *B. COLI*.

Hydrochloric acid and sulphuric acid were chosen as types for the study of strong mineral acids, and the experiments were carried out as described above. The water used was Boston tap water, containing before sterilization about 40 parts per million of residue, 15 parts of hardness, 0.015 part of free ammonia, and 0.144 part of albuminoid ammonia. The results are shown in Tables 1-4.

The 99 per cent killing-point with the hydrochloric acid is reached at a strength of 0.0077 normal, with 7.49 parts of dissociated hydrogen per million, and the absolute killing-point, as nearly as it can be determined, with a 0.0123 normal solution containing 11.80 parts

TABLE 1.
ACTION OF HYDROCHLORIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bacteria after 40 Min.
38.1	1.05	0.0010	98.0	1.03	40,000	00.00
77.6	2.13	0.0021	98.0	2.09	"	45.00
104.5	3.32	0.0033	97.5	3.24	"	72.85
149.0	4.08	0.0041	97.3	3.98	"	82.50
178.9	4.90	0.0049	97.2	4.76	"	97.50
281.0	7.71	0.0077	97.0	7.49	"	99.87
298.0	8.17	0.0082	96.5	7.90	90,000	99.96
377.0	10.61	0.0106	96.4	10.24	"	99.99
447.0	12.26	0.0123	96.4	11.80	"	100.00
515.0	96.3	"	100.00
590.0	96.3	"	100.00
663.0	96.2	"	100.00

TABLE 2.
ACTION OF SULPHURIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment.	Percentage Re- duction of Bacteria after 40 Min.
45.9	0.94	0.0009	93	0.87	60,000	66.20
46.5	0.95	0.0009	93	0.87	140,000	80.00
111.5	2.28	0.0023	90	2.05	"	81.43
138.3	2.82	0.0028	89	2.51	60,000	86.50
176.6	3.62	0.0036	88	3.18	"	96.25
178.2	3.64	0.0036	88	3.20	140,000	82.14
225.2	4.59	0.0046	86	3.94	60,000	96.25
257.2	5.25	0.0052	85	4.46	"	98.50
311.5	6.36	0.0064	84	5.35	140,000	97.50
375.9	7.68	0.0077	83	6.38	"	98.86
470.0	9.60	0.0096	80	7.68	"	99.92
552.0	11.30	0.0113	79	8.93	"	99.92
630.0	12.90	0.0129	78	10.05	"	99.95
692.5	14.13	0.0141	77	10.90	"	99.95
812.0	16.57	0.0166	76	12.60	"	100.00
910.0	18.57	0.0186	75	13.95	"	100.00

of dissociated hydrogen. With the sulphuric acid the 99 per cent reduction was reached at a strength of 0.0096 normal, with 7.68 parts of dissociated hydrogen, and the 100 per cent reduction at a strength of 0.0166 normal, with 12.6 parts of dissociated hydrogen. These results show a direct relation between disinfectant action and free hydrogen ions. The normal strengths of the killing solutions do not correspond very closely; 0.0077 sulphuric acid failed to do what the same strength of hydrochloric acid did, and 0.0129 and 0.0141 sulphuric acid failed to do what 0.0123 hydrochloric acid did. On the other hand, when we compare dissociated hydrogen, allowing for the greater ionization of the hydrochloric acid, the discrepancies disappear. The same concentrations of dissociated hydrogen, within

the limits of accuracy of the experiment, produced respectively the 99 per cent and the 100 per cent reduction in the two acids.

TABLE 3.
ACTION OF HYDROCHLORIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bacteria after 40 Min.
38.2	1.00	0.0010	98.2	1.02	7,050	79.20
75.3	2.06	0.0021	97.0	2.02	"	88.10
109.5	3.00	0.0030	97.8	2.94	"	99.30
148.2	4.06	0.0041	97.3	3.95	"	99.86
182.5	5.00	0.0050	97.1	4.85	"	100.00
216.8	5.02	0.0059	"	100.00
248.0	6.80	0.0068	"	100.00
275.5	7.54	0.0075	"	100.00

TABLE 4.
ACTION OF SULPHURIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bacteria after 40 Min.
48.0	0.98	0.0010	93.1	0.91	135,000	59.30
92.9	1.88	0.0019	90.5	1.70	"	92.97
130.4	2.84	0.0028	89.5	2.54	"	99.90
188.0	3.85	0.0038	87.0	3.35	"	96.10
223.0	4.55	0.0045	85.5	3.90	"	100.00

Tables 3 and 4 show that the typhoid bacillus is considerably more sensitive than the colon bacillus in its reaction to an excess of acid. The 99 per cent reduction was reached with hydrochloric acid at a strength of 0.0030 normal and 2.94 parts of dissociated hydrogen, and the 100 per cent reduction with 0.0050 normality and 4.85 parts of dissociated hydrogen. With sulphuric acid the 99 per cent reduction was reached with 0.0028 normality and 2.54 parts of hydrogen, and the 100 per cent reduction with 0.0045 normality and 3.90 parts of hydrogen. The fact that the 0.0038 normal solution of sulphuric acid showed only 96 per cent reduction is one of the abnormalities which unfortunately sometimes occur in bacteriological work. In general, the results show again that the two acids exert the same quantitative effect, although in this case, the solution being weaker and the dissociation of the two acids more nearly the same, the difference between normal strength and concentration of dissociated hydrogen is not clearly shown.

The critical points derived in these tests are brought together in Table 5. They show that the typhoid bacillus is a little less than half as resistant as the colon bacillus to dilute acids, and that the toxicity of these acids depends, not on their normal strength of acid or on the kind of acid used, but on the number of dissociated hydrogen ions. Between 7.4 and 7.7 parts of dissociated hydrogen effects a 99 per cent reduction of the colon bacillus, and between 11.8 and 12.6 parts, a 100 per cent reduction. For the typhoid bacillus the corresponding figures are 2.5-3.0 parts and 3.9-4.9 parts. Since at the dilutions used the hydrochloric acid was over 96 per cent dissociated, its effect must have been almost entirely ionic; and since the sulphuric acid at 75 per cent dissociation showed only the toxicity which would have been expected from its dissociated hydrogen, it appears that in this case too the undissociated molecule exerts no appreciable influence. The anions have been shown to be neutral in the experiments of other observers. It is evident, then, that the toxicity of these acids at high dilution is a function of the dissociated hydrogen.

TABLE 5.
DISINFECTANT ACTION OF MINERAL ACIDS IN TAP WATER.

	<i>B. coli</i>				<i>B. typhi</i>			
	99% Reduction		100% Reduction		99% Reduction		100% Reduction	
	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄
Normality.....	0.0077	0.0096	0.0123	0.0166	0.0030	0.0028	0.0050	0.0045
Parts per 1,000,000 dissociated hydrogen.....	7.49	7.68	12.80	12.60	2.94	2.54	4.85	3.90

4. THE DISINFECTANT ACTION OF ACETIC ACID AND BENZOIC ACID UPON *B. TYPHI* AND *B. COLI*.

We next desired to study examples of the incompletely dissociated organic acids. Acetic and benzoic acids were selected as types, and the experiments were carried out as before. The results obtained with benzoic acid are probably somewhat inaccurate on account of the difficulty of securing complete solution. The results are shown in Tables 6-8.

An inspection of these tables shows a marked difference from the results obtained with the mineral acids. With *B. coli* in acetic acid the 99 per cent reduction is reached at a strength of 0.0812 normal, and

TABLE 6.
ACTION OF ACETIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
111.3	1.85	0.0018	60,000	00.00
333.0	5.54	0.0055	6.40	0.35	"	00.00
552.0	9.18	0.0092	4.50	0.41	"	16.67
732.0	12.20	0.0122	3.50	0.43	"	23.33
1,095.0	18.25	0.0182	3.05	0.50	"	38.33
1,380.0	23.20	0.0232	2.75	0.64	"	51.67
1,825.0	30.41	0.0304	2.45	0.75	"	55.00
2,260.0	37.70	0.0377	2.20	0.83	"	50.67
3,081.0	51.40	0.0514	1.90	0.98	"	58.33
3,698.0	61.70	0.0617	1.70	1.05	"	63.33
4,800.0	80.20	0.0802	1.50	1.20	"	91.00
4,875.0	81.25	0.0812	1.50	1.21	90,000	99.99
5,610.0	93.50	0.0935	1.35	1.26	"	100.00

TABLE 7.
ACTION OF BENZOIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
237.9	1.95	0.0019	16.0	0.31	70,000	00.00
337.0	2.76	0.0028	13.6	0.38	"	50.00
406.0	3.33	0.0033	12.5	0.41	"	60.75
675.0	5.54	0.0055	9.3	0.52	"	67.80
1,184.0	9.72	0.0097	7.5	0.73	"	99.99
2,425.0	19.90	0.0199	5.4	1.07	100,000	100.00

TABLE 8.
ACTION OF BENZOIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
29.23	0.24	0.0002	40.0	0.10	50,000	44.00
140.30	1.15	0.0011	20.3	0.23	"	54.00
243.80	2.00	0.0020	15.4	0.31	"	62.00
333.00	2.73	0.0027	13.6	0.37	"	70.00
427.00	3.52	0.0035	10.4	0.37	"	92.00
689.00	5.75	0.0057	9.8	0.50	"	100.00
1,292.00	10.60	0.0106	7.2	0.76	"	100.00

the 100 per cent reduction at 0.0935 normal. The acid at these strengths is only a little over 1 per cent dissociated, and the amount of dissociated hydrogen present a little over 1.2 parts per million. Since this is only about one-sixth the strength of ionic hydrogen necessary to produce similar results with the mineral acids, it is evident that the toxic action of the acetic acids is due chiefly to the anion or the undissociated molecule, the latter, being so much greater in

amount, probably playing the principal part. The same thing is true of benzoic acid. Here the molecule is more highly toxic, producing a 99 per cent reduction at 0.0097 normality and a 100 per cent reduction at 0.0199 normality, with about 1 per cent dissociation. As in the case of the mineral acids *B. typhi* is more sensitive than *B. coli*, showing 100 per cent reduction with benzoic acid at a strength of 0.0057 normal.

It appears, then, that the toxicity of these organic acids is due, not mainly to hydrogen ions, but to the action of the undissociated molecule, varying widely, as might be expected, with the acid employed. A comparison of the corresponding toxic normal

TABLE 9.
TOXICITY OF ORGANIC AND MINERAL ACIDS FOR *B. coli* AND *B. typhi*. STRENGTH IN NORMALITY
PRODUCING 99 PER CENT AND 100 PER CENT REDUCTION.

Acid	<i>B. coli</i>		<i>B. typhi</i>	
	99% Reduction	100% Reduction	99% Reduction	100% Reduction
Hydrochloric.....	0.0077	0.0123	0.0030	0.0050
Sulphuric.....	0.0006	0.0166	0.0028	0.0045
Acetic.....	0.0812	0.0935
Benzoic.....	0.0097	0.0199	0.0057

strength, made in Table 9, shows that benzoic acid is almost as toxic as the mineral acids, the effect being due in one case to the whole molecule, and in the other case to hydrogen ions. Acetic acid, on the other hand, has only 10-20 per cent as high a disinfectant action.

5. THE DIMINISHED TOXICITY OF ACIDS IN THE PRESENCE OF PEPTONE.

Having fixed with some precision the killing-point for the various acids studied, when acting in tap water, we next desired to determine what would occur in the presence of organic matter. A series of experiments was carried out, parallel to those reported above, except that a 1 per cent solution of Witte's peptone was used instead of tap water. The results with the mineral acids, presented in Tables 10-12, showed that the toxicity of the acid is profoundly modified by the presence of organic matter.

TABLE 10.

DISINFECTANT ACTION OF HYDROCHLORIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,118	30.62	0.0306	94.8	29.0	90,000	40.00
1,825	50.00	0.0500	94.8	47.5	"	93.35
2,502	69.00	0.0690	93.0	64.2	"	97.97
2,950	80.80	0.0808	92.0	74.4	"	97.74
3,470	95.20	0.0952	91.5	87.5	"	99.98
3,685	97.80	0.0978	91.4	89.4	"	100.00
4,020	110.00	0.1100	"	100.00

TABLE 11.

DISINFECTANT ACTION OF SULPHURIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c. c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
970	140,000	00.00
1,204	"	00.00
1,405	"	00.00
1,536	31.4	0.0314	70.0	22.0	"	00.00
1,728	35.3	0.0353	68.5	24.2	"	20.00
1,962	40.1	0.0401	67.5	27.1	"	61.43
2,066	40.9	0.0409	67.0	27.4	"	64.29
2,399	48.0	0.0480	65.4	32.0	60,000	76.67
2,639	53.8	0.0538	64.8	24.9	"	79.17
2,912	59.4	0.0594	64.2	38.1	"	91.67
3,065	62.6	0.0626	63.2	30.6	"	93.33
3,258	66.5	0.0665	62.8	41.8	"	94.17
3,450	70.4	0.0700	61.9	43.5	"	98.85
4,610	94.2	0.0942	58.9	55.5	65,000	99.99
5,298	108.1	0.1081	57.0	61.6	"	100.00
6,555	135.2	0.1352	"	100.00
7,800	159.3	0.1593	"	100.00

TABLE 12.

DISINFECTANT ACTION OF HYDROCHLORIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,107	30.3	0.0330	94.8	28.7	50,000	99.99
1,740	47.6	0.0476	93.8	44.6	50,000	100.00

In these tables the dissociation values given are those determined for distilled water, and not those which actually obtain in a peptone solution. The amount of dissociated hydrogen required for disinfection, when estimated in this way, is seen to be nearly 10 times as great as in tap water. For comparison the results are brought together in Table 14.

TABLE 13.
DISINFECTANT ACTION OF SULPHURIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c. c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
819	17.03	0.0170	74.5	12.7	85,000	00.00
960	20.00	0.0200	73.7	14.7	"	17.65
1,104	22.52	0.0225	72.0	16.2	"	17.65
1,139	23.17	0.0232	72.0	16.7	"	34.10
1,288	26.32	0.0265	71.0	18.7	"	61.20
1,453	29.63	0.0296	70.2	20.8	28,000	82.15
1,472	30.03	0.0300	70.1	21.0	85,000	76.45
1,519	31.66	0.0317	69.7	21.6	28,000	89.30
1,587	32.39	0.0324	69.5	22.5	"	89.30
1,612	32.89	0.0329	69.0	22.7	"	100.00
1,678	34.24	0.0342	68.5	23.4	"	97.86
1,879	38.32	0.0383	68.0	26.0	"	100.00
1,994	"	100.00
2,045	"	100.00
2,115	"	100.00
2,119	"	100.00
2,399	"	100.00

TABLE 14.
COMPARATIVE TOXICITY OF MINERAL ACIDS IN DISTILLED WATER AND 1 PER CENT PEPTONE SOLUTION.
(Parts per 1,000,000 of dissociated hydrogen.)

	<i>B. coli</i>				<i>B. typhi</i>			
	99% Reduction		100% Reduction		99% Reduction		100% Reduction	
	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl	HO ₄
Distilled water.....	7.49	7.68	11.80	12.60	2.94	2.54	4.85	3.90
1 per cent peptone.....	87.5	55.5	89.4	61.6	28.7	44.6	22.7

It is evident that in some way the peptone exerts a strong influence in counteracting the toxic effect of the acids. It at first occurred to us that this might be due simply to the fact that peptone solution furnished a more favorable medium for the bacteria, and thus enabled them to resist unfavorable conditions. Such an effect would, however, hardly be expected in so short a period as 40 minutes; and this explanation fails to account for the fact that the toxicity of the hydrochloric acid is much more diminished than that of the sulphuric acid. Reference to Tables 15-17, which show the results obtained with the organic acids, makes it still clearer that a specific chemical action is involved.

Evidently with the organic acids disinfectant power is much less affected by the presence of peptone. With *B. coli* acetic acid produces a 100 per cent reduction when in a strength of 0.0935

TABLE 15.

DISINFECTANT ACTION OF ACETIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissocia- tion	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
4,350	72.5	0.0725	1.60	1.16	50,000	00.00
4,750	79.3	0.0793	1.50	1.19	"	00.00
5,340	89.0	0.0890	1.40	1.25	"	25.00
5,975	97.0	0.0970	1.30	1.26	"	66.00
5,995	98.0	0.0980	1.30	1.27	"	61.72
6,861	114.2	0.1142	1.25	1.42	65,000	98.75
7,540	125.8	0.1258	1.15	1.45	"	93.30
8,360	139.5	0.1395	1.05	1.46	"	100.00
9,125	152.1	0.1521	1.03	1.57	"	100.00
16,475	174.5	0.1745	1.00	1.75	"	100.00
10,720	178.8	0.1788	1.00	1.78	"
.....	210.0	0.2100	"
14,620	244.0	0.2440	0.90	2.20	"	100.00

TABLE 16.

DISINFECTANT ACTION OF BENZOIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissocia- tion	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
1,638	13.18	0.0132	6.4	0.84	70,000	28.6
1,720	14.11	0.0141	6.2	0.87	70,000	28.6
2,105	17.20	0.0172	5.8	1.00	100,000	0.0
3,305	27.60	0.0276	5.2	1.43	100,000	73.0
5,555	47.30	0.0473	3.2	1.51	100,000	100.0

TABLE 17.

DISINFECTANT ACTION OF BENZOIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissocia- tion	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Re- duction of Bac- teria after 40 Min.
1,434	11.72	0.0117	6.9	0.81	50,000	20.00
1,562	12.80	0.0128	6.6	0.84	50,000	20.00
2,155	17.80	0.0178	5.7	1.01	56,000	81.43
2,917	24.30	0.0243	4.9	1.19	56,000	100.00

normal. In the presence of peptone the required strength is 0.1395 normal. With *B. coli* the corresponding 100 per cent reduction strengths for benzoic acid are 0.0199 in water and 0.0473 in peptone solution. With *B. typhi* the respective strengths of benzoic acid are 0.0057 and 0.0243.

In a general way we may say that the presence of 1 per cent peptone solution diminishes the toxicity of hydrochloric acid, measured in terms of dissociated hydrogen, to from one-eighth to one-tenth its water value, and that of sulphuric acid to from one-fifth to one-eighth

its water value. The toxicity of benzoic acid, measured in normality, is diminished under the same conditions to from one-fourth to one-half, and that of acetic acid to a little over one-half its water value.

The most probable explanation of this phenomenon is the formation of a loose compound between the proteid molecules and the acids which would diminish the toxicity of the latter, just as Stiles and Beers (1905) have shown that such a combination alters the effect of mineral salts on muscle.

Bugarzky and Liebermann (1898), Cohnheim and Krieger (1900), and other observers, have proved the existence of such loose compounds between proteids and acids by freezing-point determinations. We desired, however, to examine the actual substance used in our own experiments. Through the kindness of Dr. Raymond Haskell, of the Research Laboratory of Physical Chemistry of this Institute, determinations of electrical conductivity were made on the peptone solution used in our experiments, on a solution of hydrochloric acid in distilled water, and on a solution of the same strength in the peptone solution.

The specific conductivity of the peptone solution was 0.0004, showing that it was fairly pure. That of the hydrochloric acid solution 0.02 normal or 720 parts HCl per million, 90.46 per cent dissociated, was 0.007. The 1 per cent peptone solution containing 0.02 normal hydrochloric acid gave a conductivity of 0.002, showing that approximately four-fifths of the hydrochloric acid had been neutralized by the peptone.

It is evident that the effect of the peptone in decreasing the toxicity of the hydrochloric acid may be explained by the fact that the number of dissociated hydrogen ions is decreased by the peptone in the same degree. The effect would naturally be less marked, as we find to be the case, with sulphuric acid, since this acid is less ionized to start with. Finally, the un-ionized organic acids are least affected. The decreased toxicity which does occur with them may perhaps be due to a loose compound with their whole-molecule—what Stiles and Beers call a “physiological compound.”

6. GENERAL CONCLUSIONS.

It appears from our experiments that the typhoid bacillus is highly sensitive to an excess of acid, being destroyed in an aqueous suspen-

sion by 40 minutes' exposure to a 0.005 normal solution of either hydrochloric, sulphuric or benzoic acid. The colon bacillus will endure exposure, under similar conditions, to solutions from two to four times as strong. Ninety-nine per cent of the bacteria in a suspension are killed by solutions of from one-half to two-thirds this strength, the last few organisms being especially resistant.

The mineral acids, hydrochloric and sulphuric, are fatal in concentrations at which they are highly dissociated. Their action runs parallel, not to their normal strength, but to the number of free hydrogen ions per unit volume. With the two organisms tested, both the 99 per cent and the 100 per cent reductions were affected, at the same concentration of dissociated hydrogen, whichever acid was used.

The organic acids, acetic and benzoic, are fatal to the typhoid and colon bacilli at a strength at which they are only slightly dissociated. The effect here appears to be due to the whole-molecule and is specific for each acid, acetic having only 10-20 per cent the toxicity of benzoic.

The presence of 1 per cent of peptone greatly diminishes the toxic action of acids, the action being somewhat less marked with sulphuric acid than with hydrochloric, and still weaker with the organic acids. In the case of hydrochloric acid we find that the diminished toxicity is accounted for by decreased ionization.

It is evident that the action of organic matter and other neutral substances in decreasing toxicity greatly complicates the study of disinfectant action. It will be necessary to bear this phenomenon in mind in considering the composition of culture media, since the apparent acidity, as determined by titration, may be quite different from the effective acidity which influences living organisms. With the mineral acids, any factor which affects dissociation, such as the presence of neutral salts of the same anion, will change the effective acidity. In considering the viability of disease germs in sewage and water, it is evident that differences in dilution and the effect of inorganic salts, organic matter, and suspended solids introduce such complex factors that detailed studies of specific local conditions are desirable.

REFERENCES.

- BECKMANN, J. W. 1896. Ueber den Einfluss des Zusatzes von Chlornatrium auf die Wirkung des Phenols. *Centralbl. f. Bakt.*, Abth. I, 20, p. 577.
- BEHRING. 1890. Ueber Desinfection, Desinfectionsmittel und Desinfectionsmethoden. *Ztschr. f. Hyg.*, 9, p. 395.
- BIAL, M. 1897. Ueber den Mechanismus der Gasgährungen im Magensaft. *Arch. f. exper. Path. u. Pharm.*, 38, p. 1.
- BIAL, M. 1902. Ueber die antiseptische Funktion des H-Ions verdünnter Säuren. *Ztschr. f. phys. Chemie.*, 40, p. 513.
- BUGARSKY, S., AND LIEBERMANN, L. 1898. Ueber das Bindungsvermögen eiweissartiger Körper für Salzsäure, Natriumhydroxid und Kochsalz. *Arch. f. d. ges. Physiol.*, 72, p. 51.
- CLARK, J. F. 1899. On the Toxic Effect of Deleterious Agents on the Germination and Development of Certain Filamentous Fungi. *Bot. Gazette*, 28, p. 289; also, *Jour. Phys. Chem.*, 3, p. 203.
- COHEN, E. 1903. Physical Chemistry for Physicians and Biologists. Trans. by M. H. Fischer, New York, 1903.
- COHNHEIM, O., AND KRIEGER, H. 1900.- Das Verhalten der Eiweisskörper zu Alkaloidreagentien zugleich eine Bestimmung der gebundenen Salzsäure. *Ztschr. f. Biol.*, 40, p. 95.
- DRESER, H. 1893. Zur Pharmakologie des Quecksilbers. *Arch. f. exper. Path. u. Pharm.*, 32, p. 456.
- FROST, W. D., AND SWENSON, M. W. 1906. Note on the Thermal Death-Point of *B. dysenteriae* Shiga. *Science*, N. S., 23, p. 216.
- GAGE, S. D., AND STOUGHTON, G. VAN E. 1906. A Study of the Laws Governing the Resistance of *B. Coli* to Heat. *Science*, N. S., 23, p. 216.
- HAMBURGER, H. J. 1904. Osmotischer Druck und Ionlehre in den medicinischen Wissenschaften. Wiesbaden, 1904.
- HEALD, F. D. 1896. On the Toxic Effect of Dilute Solutions of Acids and Salts upon Plants. *Bot. Gazette*, 22, p. 125.
- HEIDER, A. 1892. Ueber die Wirksamkeit der Desinfectionsmittel bei erhöhter Temperatur. *Arch. f. Hyg.*, 15, p. 341.
- JOHNSON, G. A. 1905. The Use of Copper Sulphate for Reducing the Pathogenicity of Sewage and Sewage Effluents. *Jour. New Eng. Waterworks Assoc.*, 19, p. 506.
- KAHLENBERG, L. 1898. The Action of Solutions on the Sense of Taste. *Bull. University of Wisconsin*, 2, p. 1.
- KAHLENBERG, L., AND TRUE, R. H. 1896. On the Toxic Action of Dissolved Salts and Their Electrolytic Dissociation. *Bot. Gazette*, 22, p. 81.
- KOCH, R. 1881. Ueber Desinfection. *Mith. a. d. kaiserlichen Gesundheitsamte*, 1, p. 1.
- KRÖNIG, B., AND PAUL, T. 1897. Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfection. *Ztschr. f. Hyg.*, 25, p. 1.
- LOEB, J. 1897. Physiologische Untersuchungen über Ionenwirkungen, I. *Arch. f. d. ges. Physiol.*, 69, p. 1.
- LOEB, J. 1898. Physiologische Untersuchungen über Ionenwirkungen, II. *Arch. f. d. ges. Physiol.*, 71, p. 457.

- LOEB, J. 1905. Studies in General Physiology. *Decennial Publications of the University of Chicago*, Chicago, 1905.
- MAILLARD, L. 1898. Du rôle de l'ionisation dans les phénomènes vitaux. *Comptes rend. de la Soc. de Biol.*, 50, p. 1210.
- MAILLARD, L. 1899. Rôle de l'ionisation dans la toxicité des sels métalliques; sulfate de cuivre et *Penicillium glaucum*. *Bull. Soc. chim. Paris*, 21, p. 26.
- MINERVINI, R. 1898. Ueber die baktericide Wirkung des Alkohols. *Ztschr. f. Hyg.*, 29, p. 117.
- NÄGELI, C. VON. 1893. Ueber oligodynamische Erscheinungen in lebenden Zellen. *Neue Denkschriften der allgemeinen Schweizerischen Gesells. f. d. ges. Naturwissenschaften*, 33, Abth. 1.
- PAUL, T., AND KRÖNIG, B. 1896. Ueber das Verhalten der Bakterien zu chemischen Reagentien. *Ztschr. f. phys. Chemie*, 21, p. 414.
- RÖMER, C. 1898. Ueber Desinfection von Milzbrandsporen durch Phenol in Verbindung mit Salzen. *Münch. med. Wchnschr.*, 45, p. 298.
- SCHEURLÉN. 1895. Die Bedeutung des Molecularzustandes der wassergelösten Desinfectionsmittel für ihren Wirkungswerth. *Arch. f. exper. Path. u. Pharm.*, 37, p. 74.
- SCHEURLÉN AND SPIRO. 1897. Die gesetzmässigen Beziehungen zwischen Lösungszustand und Wirkungswerth der Desinfectionsmittel. *Münch. med. Wchnschr.*, 44, p. 81.
- SEDGWICK, W. T., AND WINSLOW, C.-E. A. 1902. Experiments on the Effect of Freezing and Other Low Temperatures upon the Viability of the Bacillus of Typhoid Fever, with Considerations, Regarding Ice as a Vehicle of Infectious Disease. *Memo. Amer. Acad. Arts and Sciences*, 12, p. 471.
- SPIRO, K., AND BRUNS, H. 1898. Zur Theorie der Desinfection. *Arch. f. exper. Path. u. Pharm.*, 41, p. 355.
- STEVENS, F. L. 1898. The Effect of Aqueous Solutions upon the Germination of Fungus Spores. *Bot. Gazette*, 26, p. 377.
- STILES, P. G., AND BEERS, W. H. 1905. On the Masking of Familiar Ionic Effects by Organic Substances in Solutions. *Amer. Jour. Physiol.*, 14, p. 133.
- TRUE, R. H., AND OGLEVEE, C. S. 1905. The Effect of the Presence of Insoluble Substances on the Toxic Action of Poisons. *Bot. Gazette*, 39, p. 1.
- WÜTHRICH. 1892. Ueber die Einwirkung von Metallsalzen und Säuren auf die Keimfähigkeit der Sporen einiger der verbreitetsten parasitischen Pilze unserer Culturpflanzen. *Ztschr. f. Pflanzenkrankheiten*, 2, p. 17.

A CONTRIBUTION TO THE GENERAL PRINCIPLES OF THE PHARMACODYNAMICS OF SALTS AND DRUGS.*

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THIS paper is a continuation of those already published,¹ which have had for their object the investigation of the means by which salts and drugs influence the processes going on in living matter, and thus produce the phenomena of stimulation and depression.

PART I. PHARMACODYNAMIC ACTION DUE TO IONS.

The cause of the pharmacological action of salts upon protoplasm has been the subject of numerous investigations, but until the development of the ionic theory these investigations had led to no further result than to show that in groups of similar metals the heavier were frequently the more poisonous. The application of the ionic theory first brought some order into this part of pharmacology. The work of the American investigators Kahlenberg and True² and Heald, confirmed as it has been by Krönig and Paul, Höber, True, and many others, has shown in the clearest manner that there is a close parallelism between toxicity and the state of ionization of many of the metals, so that these authors conclude that the pharmacological action of any salt solution is a function, in large measure at least, of the ions into which the salt dissociates.

This general conclusion is, in my opinion, as firmly established as is the conclusion that the chemical reactions of such solutions are due to the ions they contain. Indeed, the conclusion is a necessary result of the ionic theory, since the chemical reactions in protoplasm do not differ in nature from those going on elsewhere; and if salts enter into other chemical reactions by their ions, they probably enter also in the same manner into the reactions of protoplasm.

But while this general theory is a great step forward, it stumbles against the objection that many compounds profoundly affect proto-

* Received for publication March 23, 1906.

¹ MATHEWS, *Amer. Jour. Physiol.*, 1904, 10, p. 291; 1904, 11, p. 455; 1905, 14, p. 204; 1905, 12, p. 421; 1904, 11, p. 238.

² KAHLENBERG AND TRUE *Botanical Gazette*, 1896, 22, p. 91.

plasm, although they do not dissociate electrolytically into ions in the ordinary sense of the term. To explain the action of such compounds as ether and organic drugs, either one must fall back upon the assumption of the action of undissociated molecules, or the idea of dissociation must be extended to cover dissociation which is not accompanied by electrical conductivity. Kahlenberg and True, and indeed nearly all observers, have adopted the theory that some action must be ascribed to undissociated molecules; but it appears to me, in view of the fact that such another kind of dissociation is well known to occur—as, for example, the dissociation of NH_4OH into NH_3 and H_2O —and also that this dissociation has been shown by Nef¹ to determine the chemical reactions of such compounds, that the alternative of the action of dissociated particles is the more probable. At any rate, it would be premature to ascribe pharmacological action to undissociated molecules until the possibilities that that action is due to the dissociated particles shall have been proved to be insufficient. In the present paper I shall deal with pharmacological action due to particles dissociated as ions, and in a subsequent paper to action due to non-ionic dissociation. The general principles which I have worked out apply primarily to ionic particles, but I think it altogether probable that they will be found to apply equally well to non-ionic dissociation, since there is in all likelihood no essential difference in kind between such dissociation as that of NH_4OH into NH_3 and H_2O and ionic dissociation. The two probably differ only in that in the one case the two electrical charges are on the same particle, whereas in ionic dissociation they are on separate atoms.²

While, then, it cannot be denied that some action may be referable to undissociated molecules, the clear parallelism between dissociation and pharmacological action in the case of salts, and the equally clear parallelism between non-ionic dissociation and pharmacological action in organic compounds, indicates to my mind that it is to these dissociated particles as the possible cause of pharmacodynamic action that attention should first be directed.

Assuming, therefore, that the action of salts is due in chief measure to the ions of the solution, the first question to be answered is:

¹ NEF, *Liebig's Annalen*, 1904, 335, p. 192.

² MATHEWS, *Biological Bulletin*, 1905, 8, p. 342. See also NERNST, *Theoretische Chemie*, 4th ed., 1903, p. 378.

What enables any ion to act at all? What makes a mercury ion, for example, so enormously more toxic than a calcium or magnesium ion? The answer to this question, as I shall now proceed to show, is, that the mercury ion has an enormously greater ionic potential than the calcium ion.

IONIC POTENTIAL AND PHYSIOLOGICAL ACTION.

In an earlier paper¹ the term "ionic potential" was suggested to designate the tendency of any ion or atom to change its electrical state. Bodländer has used the term "Haftintensität" to designate the same factor, and he and Abegg have presented evidence to show that the ionic potential is one of the chief factors in determining chemical affinity.

The idea that this property of the ions of the salt might be of importance in determining their physiological action was first suggested by my colleague, Dr. J. Stieglitz, at the meeting of the American Physiological Society in Chicago in December, 1901. At that time the importance and real bearing of the suggestion were not appreciated by me, but about a year or so later I was much struck by the fact that the arrangement of the metals according to their solution tensions, as given by Nernst, was practically the same as an arrangement in the order of their toxic actions. Stieglitz's suggestion appeared to me in a new light, and I set to work to get additional evidence that it is the ionic potential which chiefly determines the physiological action of ions. In 1904 I published results showing the remarkable parallelism between toxicity and ionic potential in the action of salts on the eggs of *Fundulus heteroclitus*. In that paper I showed that valence and ionic velocity—factors to which main importance had been attached by Hardy, Loeb, Pauli, Posternak, and which have been recently emphasized by Robertson²—are unimportant when compared with the importance of the ionic potential as a determining factor of toxicity. I showed also that the phenomena of stimulation of the motor nerve by salts demonstrate the same relationship over again, and in the clearest and most decisive manner. Inasmuch as I had already interpreted the phenomena of chemical stimulation of motor nerves to mean that the nerve impulse was due to a progressive coagulation of the colloids of the nerve, it was a

¹ MATHEWS, *Amer. Jour. Physiol.*, 1904, 11, p. 456.

² ROBERTSON, *Trans. Roy. Soc. of South Australia*, 1905, 29, p. 11.

necessary inference, if this were true, that the ionic potential must be of decisive value in determining the precipitation of colloids by electrolytes. An investigation of this possibility showed that this was indeed the case. McGuigan¹ then investigated the relation between the ionic potential and the power of salts to prevent the action of the diastatic ferment upon starch, and found here also a remarkably close agreement with the theoretical anticipations.

The theory of the importance of the ionic potential has, therefore, been abundantly confirmed. It is the more surprising that it has met with little acceptance or attracted little notice, since its general bearings are exceedingly important, involving as they do the nature of chemical affinity on the one hand, and the basis of pharmacology on the other.

Owing to the importance of the subject, the slight attention it has received, and to the fact that my own ideas have become during the course of the work more clear and definite, it seemed to me desirable that the results previously presented both by myself and by others be summarized and put in a more definite, and perhaps a more comprehensible, form, together with new observations in the same direction. Since the solution tension and ionic potential are properties with which physiologists are not generally very familiar, since they lie in another field not hitherto brought into relationship with physiological processes, I have tried to get these ideas clear at the outset.

a) *General physical principles involved in chemical stimulation and toxicity.*—Any physiological response to an external agent, however that response is produced, implies a change in motion or in state of the atoms, molecules, and masses composing the protoplasmic system. Now such a change in state means that work has been done in producing these movements, and this work must have been done at the expense either of the internal energy of the system itself, or of the energy of the environment—in this case of the substance causing the change. There are accordingly two possible ways in which an external agent such as a salt might produce a change in the protoplasmic system. It may itself supply the energy, in whole or in part, which is necessary to bring to pass the internal movements of the system; or it may by its presence facilitate the transference of the potential

¹ MCGUIGAN, *Amer. Jour. Physiol.*, 1904, 10, p. 444.

energy of the system itself into kinetic. The first method of action is clear, but a word may be said as regards the second. Protoplasm, both in its chemical and physical aspects, shows many of the phenomena of false equilibrium. It is as if there were considerable differences of potential in the protoplasm itself, but these differences were unable to neutralize or equalize themselves, owing to the presence of certain resistances. It is conceivable that our ions may produce results simply by acting as conductors, or in removing resistances; acting, in other words, as catalytic agents, without specifying more in detail exactly how these act. As an example of this kind of an action I may mention the generation of the nerve impulse when a motor nerve is suddenly immersed in a salt solution, or when its cut and longitudinal surfaces are connected by a wire. In this case the wire or the electrolyte serves by its presence only to equalize the difference in potential between the two surfaces, and the nerve stimulates itself by its own energy. And any electrolyte or any conductor will accomplish this result. To what extent electrolytes may thus affect protoplasmic motions cannot be foretold, but it is certainly possible, and I think on the whole probable, that some of the actions of salts will be found to be of this nature. In such cases the energy content of the salt would be of little importance. But while it cannot be denied that some of the salt action may be of this character, few specific instances are known to me.

In the second place, salts may appear to act catalytically by means of their valence by bringing about combinations between two substances, this combination resulting in one substance hastening the decomposition of the other. The ferments, for example, may in this way be mordanted, as it were, by some bivalent ions to the substances they ferment, in the manner suggested by Henri.¹ It will, however, be apparent in this case that the power of the ion to form such combinations of the right degree of looseness from which the ferment can again escape, must be dependent on the chemical affinity of the ion. Since the chemical affinity is very probably a function of the ionic potential, this case also really brings us back to the ionic potential as a highly important factor in the ion's action.

We may now turn from these hypothetical cases to the other possi-

¹ HENRI, *Revue générale des sciences*, 1905, 16th year, p. 641.

bility in which salts affect the protoplasmic movements in virtue of their own energy content.

In this case also the action of the salt may be twofold. It may either change the whole protoplasmic system by means of the energy in the salt, or it may by a transfer of a portion of its energy to one part of the protoplasm produce such a change in the latter that energy is set free by the protoplasm itself. It is clear, in other words, that the salt may destroy the protoplasm either directly, in virtue of a great interchange of energy between itself and the protoplasm, or it may destroy it indirectly, by acting on some part of the protoplasm in such a way that its own energy destroys it, or that the normal conversion of potential into kinetic energy necessary for the continuance of the vital processes is checked.

A distinction is generally made between these two forms of destruction, in that substances acting in the first manner are said to be immediately fatal; those acting in the second manner are said to exhaust the protoplasm by over-stimulation or depression. Thus mercuric chloride in large doses probably produces an immediate coagulation and destruction of the living matter. In this case an immediate and complete change in the protoplasmic system would be produced by the transfer of energy from the salt to the protoplasm as a whole. On the other hand, mercuric chloride may destroy living matter in small doses, not by this method, but by bringing about a small change in the protoplasm, by means of which internal resistance of some kind is withdrawn or increased, and the protoplasm destroys itself. In both these cases, however, the destruction of the protoplasm is a direct result of the energy content of the salt, and salts will be poisonous according as the amount of free energy in them is great or small.

For all salts and compounds producing changes in the protoplasmic system in the two last ways the chemical composition will be of little or no importance; the sole or most important factor determining action will be the potential and amount of the energy in it. The character of the carrier of the energy, in other words, is immaterial.

The foregoing considerations may be expressed in a formula:

Poisonous action of any salt = work done by it = available energy in it = amount of energy \times its potential.

In the action of salts on protoplasm we have to deal, then, with a transfer of energy from the ions to the protoplasm, or vice versa. From the general principles of physics we conclude that the physiological action of any salt solution must be a function of its energy content. The question arises how this energy content is to be measured.

It has been shown that much, if not all, of the action of salt solutions is due to the ions present. We must, therefore, measure the energy content of the ions. The total energy of the ion is composed of two factors, the free or available energy and the bound energy. It is only the free energy, or that which can be transferred to or from the ion, which is of importance in this connection.

1. *The potential factor of the free energy.*—The interchange of energy between the salt solution and the protoplasm must depend on the relative potentials of the two systems, since whether any substance can transfer energy to another depends, not on the total amount of energy in the two substances or systems, but on the potential of the energy in the two cases. The action of any salt solution is then determined by its *available* energy, and by the *available* energy in any salt is meant the product of the difference of potential between the protoplasm and the salt multiplied into the amount of energy transferred from one to the other before the potential is equalized. If the protoplasm and the ion have energy at the same potential, the difference in potential will be zero, the available energy is hence zero, the work done is zero, and the ion should produce no direct effect due to its energy content on protoplasm, though it might affect it catalytically in the manner indicated.

2. *Total free energy.*—The total free or available energy of any ion is composed of two factors, the potential energy and the kinetic energy. The *kinetic energy*, or energy of motion, will be equal to $\frac{1}{2} MV^2$. As I do not know the actual velocity of ionic movement when the potential gradient is unknown, I am unable to determine the kinetic energy. It is, in any case, generally small when compared to the potential energy, although not negligible when the latter factor approaches zero. That is, if the potential of two ions and the protoplasm are about the same, these ions may have different actions owing to differences in their kinetic energy, i. e., their ionic masses and velocities. In this paper, however, I shall consider only the potential energy factor.

3. *The potential energy of ions.*—What is the measure of the potential energy of any ion? The potential energy must be the difference in the energy content of the ion or atom in different conditions. If any substance has any available potential energy, it necessarily means that it is capable of existing in two conditions which differ in their energy content, and that it gives up energy in passing from one condition to the other. That ions and atoms do exist in such different conditions is well known. Thus the chemical differences between atomic and ionic sodium, and between ferric, ferrous, and metallic iron, are due to differences in the energy content of the atoms in different conditions. The available potential energy of the sodium atom is very much greater than that of the sodium ion, as is indicated by the fact that when the atom becomes an ion, a large amount of heat is set free.

The potential energy of the ion must be sharply distinguished from the ionic potential. The potential energy is in its turn composed of a capacity and an intensity factor; the capacity factor being represented by the amount of electricity transferred; the intensity factor, by the tendency of the ion or atom to change its state; in other words, by its stability or ionic potential. The potential energy of any ion must be measured hence by the ionic potential multiplied into the capacity. The capacity factor falls out of account if equivalent solutions are compared, since in that case each equivalent has the same quantity of electricity in it, and the differences between the actions of ions are hence due to differences in ionic potential. The question now comes down to the determination of the ionic potential.

4. *The determination of the ionic potential.*—In my earlier papers it was not clear to me how this ionic potential could be determined, so I used instead, as a rough measure of it, the solution tension of the metal. I assumed that the solution tension would be the reciprocal of the ionic potential. Inasmuch as the solution tension varies with the concentration of the salt, I used arbitrarily the solution tension of the metals in normal ionic solution.

Inasmuch as the determination of the ionic potential depends on the determination of the solution tension, it is necessary to understand the latter term, and as this may not be familiar to all physiologists, the following explanation is given:

TABLE 1.
SOLUTION TENSION IN VOLTS OF ELEMENTS IN NORMAL IONIC SOLUTIONS.

Cations				Anions	
K	-2.92	Zn	-0.493	Cl	-1.694
Na	-2.54	Cd	-0.143	Br	-1.270
Ba	-2.54	Fe''	-0.063	I	-0.797
Ca	-2.28	Co	+0.045	NO ₃	-2.229
	-1.88(?)	Ni	+0.049		
Sr	-2.49(?)	Pb	+0.129		
Li	-2.32	H	+0.277		
Mg	-2.26	Fe'''	+0.314(?)		
	-1.48(?)	Cu	+0.606		
Al	-0.999	Hg	+1.027		
Mn	-0.798	Ag	+1.048		

When a plate of metal is placed in water, a certain amount of the metal passes into the water in the form of positively charged particles, so that the solution becomes positively charged, the metal negatively charged. The tendency of different metals to throw off these positive particles varies, and this tendency may be measured in so many volts if the metal is placed in a solution of one of its salts of known strength. If this is done and the difference in voltage between the metal and solution is measured, one obtains a series of values for the different metals, and these values are known as the solution tension series of the metals. The measurements are generally referred to the metals when immersed in normal ionic solutions of their salts (Table 1).

By a reference to Table 1 showing this series it will be found that potassium and sodium stand at one end of the series, these metals having in normal ionic solutions a negative voltage of 2.92 and 2.54 respectively, as compared with the solution; while at the other end are the noble metals, gold, silver, platinum, and mercury, which immersed in normal ionic solutions become electropositive to the extent of more than one volt.

The solution tension measures therefore the difference in potential between the solution which contains a known quantity of the ions of the metal and the metal itself; and it expresses the difference between the tendency of the ion to deposit itself on the metal plate, and the tendency of an atom of the plate to become an ion. It will be seen that the values of the solution tension depend entirely on the concentration of the ions in the solution, and the presence of a plate or particle of the metal. For our purposes, therefore,

it is clear that the ordinary figures given for the solution tension are not strictly applicable to the physiological conditions. We introduce into the salt solution, not a plate of metal, but a particle of protoplasm, and we wish to know what is the tendency of any ion in that solution to give up its charge in whole or in part to the protoplasm. The solution tension is not therefore a proper measure of the particular property of the ion we seek.

In previous work, in order to get comparable results, I had to adopt arbitrarily the solution tension of the metals in the normal ionic solutions of their salts, although there was no reason at all why this value, rather than the value in any other concentration, should have been taken.

It is clear that what is most important in the ion in determining its physiological action, and its chemical action as well, is not the difference of voltage between a plate of metal and any solution of its salts, but rather the difference in pressure between a single ion and a single atom of the metal. That is, it is the inherent tendency of any ion in any concentration to change into an atom of its metal. This last property has been called the ionic potential. The method of computing it is as follows:

Nernst¹ has shown that the formula which expresses the amount of work necessary to compress a gas from volume 1 to volume 2 is of very general applicability, and also expresses the amount of work necessary to transform one gram atom of a metal into one gram ion at any concentration. This formula is as follows:

$$\text{Amount of work} = L = RT \ln \frac{v_1}{v_2}.$$

In this formula R is the gas constant; T , the absolute temperature; v_1 and v_2 the gas volumes; and the logarithm is the natural logarithm. This formula may also be expressed using pressures instead of volumes, or:

$$L = RT \ln \frac{p_2}{p_1}.$$

In this formula p_1 is what is known as the solution pressure of the metal, and p_2 the osmotic pressure of the ions of the metal in the solution. Instead of p_1 we may write P . By taking R and T in absolute

¹ NERNST, *Theoretische Chemie*, 1903, 4te Aufl.

units, and remembering that in all cases one gram ion carries $n \times 96,540$ coulombs of electricity, where n is the valence, this formula may be expressed in the form of potential in volts existing between the metal and solution,¹ i. e.:

$$E = \frac{RT}{n} \ln \frac{p_2}{P} = \frac{0.057}{n} \log_{10} \frac{p_2}{P} \text{ volts.}$$

If this potential is measured directly by connecting the metal immersed in a solution of its salt through a voltmeter with an electrode of known potential, E may be measured, and then P is easily calculated. When P is once known, E may be calculated when the metal is immersed in any solution of its salts of which p_2 is known.

To determine the real ionic potential from this formula, one proceeds as follows: It is obvious that the formula expresses the amount of work done in accomplishing two different things. It expresses the sum of the work necessary to transform one gram atom of metal into one gram ion *in the same space*, plus the amount of work (negative) necessary to expand the one gram ion from this space to one liter or the space it finally occupies. It is the first of these factors which we wish to determine, since this measures the ionic potential. The formula may accordingly be written as follows:

$$L = RT \ln \frac{p_2}{P} + RT \ln \frac{p_3}{p_2}.$$

In this formula p_2 is the osmotic pressure of the positive ions of the metal when at the same concentration as the atoms of the metal; and p_3 is the osmotic pressure of the ions when at the concentration of one gram ion to the liter. Accordingly, the first term of the right-hand member of the equation measures the work necessary to transform one gram atom of the metal into one gram ion occupying the same space, and the second term measures the work done (negative) in expanding from this space to one liter. Expressing this formula in volts, and putting C =concentration in place of p , and passing to common logarithms

$$E = \frac{0.057}{n} \log \frac{c_2}{C} + \frac{0.057}{n} \log \frac{c_3}{c_2}. \quad (1)$$

In this equation E is determined by measurement, and the second term is easily calculated. The middle term, or the ionic potential, is then

¹ *Ibid.* p. 701.

obtained by the difference between E and the second term. For example, a silver plate in contact with a normal ionic silver nitrate solution shows a difference of potential between itself and the solution of $+1.048$ volts. $\therefore E = +1.048$ volts. c_2 is the concentration of silver atoms in metallic silver. One gram atom of silver—i. e., 107.9

TABLE 2.
THE IONIC POTENTIALS OF THE IONS OF METALS IN VOLTS.

K $-2.92(?)$	Cd -0.089	Cl -0.426
Na $-2.54(?)$	Fe' $+0.00$	Cl $-1.694(?)$
Li $-2.32(?)$	Co $+0.107$	Br $-1.270(?)$
Ba $-2.54(?)$	Ni $+0.112$	I $-0.797(?)$
Ca $-2.26(?)$	Pb $+0.179$	
Sr $-2. (?)$	H $+0.107(?)$	
Mg -1.160	Cu $+0.668$	
Mn -0.737	Hg $+1.080$	
Zn -0.434	Ag $+1.163$	

grams of silver—occupies the space at 18° of 10.1 c.c.; i. e., the atomic weight in grams divided by the specific gravity. c_2 , or the number of gram atoms of silver in one liter of silver, $= \frac{1000}{10.1} \cdot c_3 = 1$, since the concentration of silver ions is one gram ion per liter. Substituting these values in (1),

$$1.048 \text{ volts} = 0.057 \log \frac{c_2}{C} + 0.057 \log \frac{10.1}{1000}$$

or

$$1.163 = 0.057 \log \frac{c_2}{C}.$$

There is hence a difference of potential of 1.163 volts between one atom of silver and one ion, in favor of the ion. That is, when one gram ion of silver changes into one gram atom *in the same space* $96,540 \times 1.163$ Joules of energy are set free, or since each monovalent ion carries 9.65×10^{-20} coulombs of electricity, when one silver ion changes into a silver atom at any concentration, $9.65 \times 10^{-20} \times 1.163$ Joules of energy are set free.

It will be seen, by comparing Tables 1 and 2, that the true values of the ionic potential calculated in this way do not in most instances differ greatly from the values of the solution tension in normal ionic solutions. The heavy metals have, as a rule, an ionic potential about $0.07-0.1$ volts different from the solution tension in normal ionic solutions.*

* I have not calculated the ionic potentials of Cl, Br, and I, but have used instead the figures for solution tension in normal ionic solution. It is also impossible to calculate the ionic potentials of Ca, Li, Ba, Na, and K.

A word may be said about the reliability of these figures. They depend, as will be seen, upon direct measurements of the electromotive force shown between two metals when immersed in known solutions of their salts, those solutions being in contact. This assumes a knowledge of the number of ions of metal in the salt solutions in question, and this factor is not in all cases perfectly certain. A much more serious source of possible error arises from a determination of the potential of any single metal, since it is necessary to know the potential of at least one electrode before the rest can be determined. The measurement is ordinarily made by using the calomel mercury electrode, which is supposed to have a potential of $+0.56$ volts. This voltage was determined by measuring the potential between a dropping mercury electrode, which theoretically should have a zero potential, and the calomel mercury electrode. Recent determinations of the potential of the calomel mercury electrode by a totally different method by Billitzer¹ give a different value. It is, therefore, impossible at present to say which of these measurements or methods gives the more reliable result. Nernst has accordingly proposed that the hydrogen electrode in normal ionic solution be regarded arbitrarily as zero, until the absolute potential is determined. I have, however, used the values as given by Wilsmore, based on the calomel electrode 0.56 .

The question does not influence most of the measurements which follow, since these are based on the sum of the potentials of both anion and cation, or upon the differences between two like ions. This is a constant whatever the absolute potential, since if a certain number is added to the anion, it will be subtracted from the cation. For example, suppose it be shown by a change in the point of zero potential that the solution tension of sodium is 2.34 instead of 2.54 , this increases the ionic potential by 0.2 volt; but if sodium is 2.34 , then chlorine is 1.894 instead of 1.694 , and this reduces the ionic potential of chlorine by 0.2 volt. The sum of the potentials of sodium and chlorine remain unchanged.

b) *The relation between physiological action and ionic potential.*—To bring out the relationship between ionic potential and toxic action, I have prepared Table 3, which shows this relationship for the toxic

¹ BILLITZER, *Ztschr. für Elektrochemie*, 1902, 8, p. 638.

action of salts on fish eggs,¹ the diastatic ferment,² bromelin,³ a proteolytic ferment, and growing tips of peas and beans.⁴ Table 3 shows that the chlorides of the various metals arrange themselves, as regards their toxicity, with few exceptions, in the order of the potential energy of their ions. Ions of low potential energy, such as those of sodium, lithium, magnesium, and potassium, being relatively inert, when compared with the enormously toxic action of the ions of high potential energy, such as nickel, lead, hydrogen, ferric, cupric, mercury, silver, gold, and platinum.

There is, I think, no mistaking this general parallelism, which was theoretically anticipated. By no other properties known to us can the metals be arranged in an order so closely corresponding to their

TABLE 3.
MINIMUM FATAL DOSES OF SALTS FOR VARIOUS FERMENTS AND ORGANISMS.
(*V* = Dilution Minimum Fatal Dose (Equivalent).)

SALT	MINIMUM FATAL DOSE (<i>V</i>) EQUIVALENT DILUTION						
	Diastase	Eggs of Fundulus	Cilia Volvox	Bromelin	Roots of Pisum	Roots of Zea mais	Roots of Lupinus
Ag NO ₃	< 100,000	100,000	300,000	110,000	204,800	300,000	300,000
Hg NO ₃	75,000
Hg Cl ₂	30,000	50,000	204,800	25,600
Hg (CN) ₂	51,200
Cu SO ₄	30,000	51,200	102,400	12,800
Cu Cl ₂	8,333	15,000	24,000	51,200	102,400	12,800
Te Cl ₂	333	4,000
Pb (NO ₃) ₂	30(?)	20,000	6,700
Pb Cl ₂	5,000	18,500
Pb (C ₂ H ₃ O ₂) ₂	5,000
HCl.....	990	3,000	12,800	3,200	3,200
Cd (NO ₃) ₂	3,000	51,200
Cd Cl ₂	142	12,500	500
Ni Cl ₂	910	500
Ni SO ₄	51,200	51,200	12,800
Co Cl ₂	100	250
Co SO ₄	2,250	12,800
Co (NO ₃) ₂	2,400	25,600	6,400	12,800
Fe Cl ₂	10
Fe SO ₄	6,400
Zn Cl ₂	69	800
Zn (NO ₃) ₂	14,000
Zn SO ₄	8,350
Mn Cl ₂	6.25	4	15
Al Cl ₃	3.3	3
Mg Cl ₂	1	2	5
Mg (NO ₃) ₂	325
Li Cl.....	1.4	4	1,000 (LiCl)
Ca Cl ₂	4	3.5	5
Ba Cl ₂	1.1	2
Lj Cl.....	2.5	1.5	400
Na Cl.....	> .3	2
K Cl.....	> .3	1.3	4(?)	100
Na NO ₃	1,600(?)
K NO ₃	100

¹ Authors' results.

² McGugan, *loc. cit.*

³ Caldwell.

⁴ Kahlenberg and True, *loc. cit.*; Heald, *loc. cit.*

toxic action. For example, suppose it was attempted to classify them in the order of their ionic weights. It will be seen that no parallelism exists between toxic action and ionic weight, since we have nickel, atomic weight 58, lead, atomic weight 200, hydrogen, atomic weight 1, and copper atomic weight 32, following each other closely. Furthermore, attention may be called to the enormous difference in toxicity, existing between the same atom when carrying two different quantities of potential energy. Ferrous iron has as an ion very little potential energy compared with ferric iron, and it is enormously less poisonous than the latter.

It will be noticed also that in each of these cases certain metals come out of their proper order of toxicity and potential. In the case of *Fundulus* eggs, cadmium is considerably out of its proper place, whereas it follows the rule in the case of diastase; for diastase, lead is quite out of its position; for bromelin, the great exception is barium, which is very toxic. The causes of these special and sporadic exceptions will be taken up later.

TABLE 4.
MINIMUM FATAL DOSE AND IONIC POTENTIAL OF ANIONS.
(Eggs of *Fundulus heteroclitus*.)

Salt	V (Min. Fatal Dose)	Anionic Potential
NaNO ₃	2.0	-2.229 (?)
NaCl	2.0	-1.694 "
NaBr	2.7	-1.270 "
NaI	4.0	-0.797 "
NaBrO ₃	11.0	-0.727 "
Na ₂ C ₂ O ₄	35.0	-0.109 "

If now we turn to the negative ions, or anions, a similar parallelism is shown to exist between toxicity and potential energy content. Thus chlorine is in all cases far less toxic than iodine, which has twice as much potential energy. The oxalates and cyanides and sulphites are the more toxic, the greater their available energy content. Unfortunately, our knowledge of the potentials of the anions is less exact than our knowledge of the potentials of the cations, so that it is impossible to follow the correspondence in detail; but sufficient is shown to prove that the same correspondence between toxicity and potential energy exists here as in the cations.

c) *The quantitative relationship between ionic potential and the minimum fatal dose.*—It was shown at the outset that the amount of

work any ion can do must depend upon its available potential energy; i. e., upon the product of the difference of potential between the protoplasm and the ion multiplied into the quantity of energy transferred before the potentials were equalized. Of two positive ions holding different quantities of available potential energy, that which has the more energy can do the more work. The total amount of work any number of positive ions can do will depend on the concentration of the the ions and amount of available potential energy in each ion. If we take as a standard a certain amount of work—let us

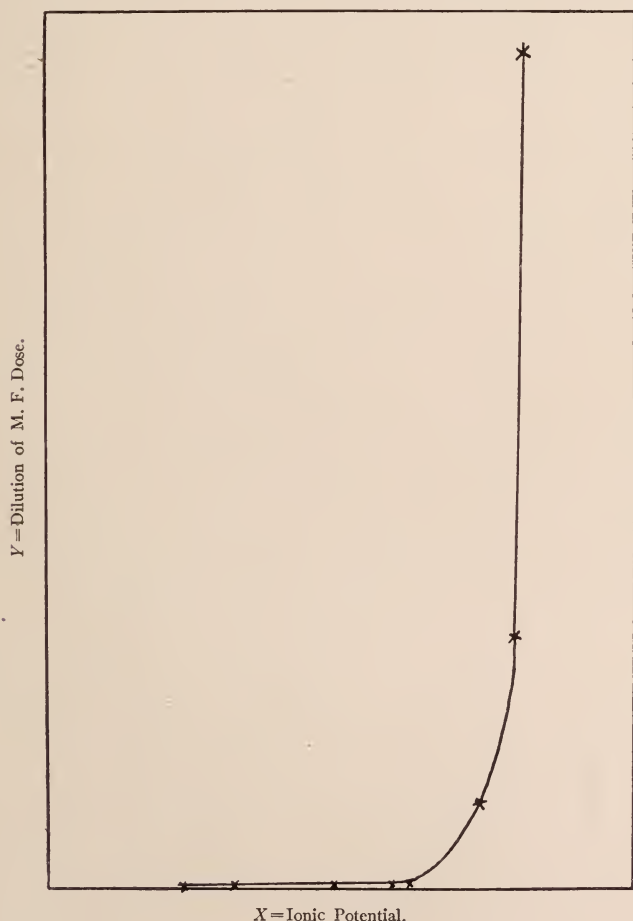


FIG. 1. Ordinates represent the dilution of the minimum fatal dose
Abscissæ represent the cation potential.

say the amount of work just sufficient to kill protoplasm in unit time, say 24 hours—the concentrations of the two ions which will just accomplish this work must stand in some numerical relation to their potential energy content. What is that relation?

To bring out this relationship, I have plotted the curve in Fig. 1, which expresses the relationship between the ionic potential of the cation and the dilution (V) of the minimum fatal dose. By dilution is meant the number of liters of solution containing one gram equivalent of the salt. From an inspection of this curve it will be seen that we are dealing with a logarithmic function; that is, the dilution increases enormously in a logarithmic ratio to the ionic potential. The dilution, for example, increases 100,000 times while the ionic potential increases about five times.

By inspection of this curve one may write the general equation:

$$\log_{10} V = KE \quad 1$$

In this formula E is the ionic potential, V the dilution, and K the constant of proportion. If, however, we place $\frac{\log V}{E} = K$, it will be seen that this formula is not in the right form, since E for some ions is zero. Nor does the formula $\log V = \frac{K}{E_a + E_c}$ give constant results. In

this formula $E^c + E^a$ represents the sum of the potentials of the anion and cation. Nor could it be anticipated that such a formula would give a proper result, since what we have to express is the difference of potential between the salt and the protoplasm, and this formula expresses only the relation between minimum fatal dose and the absolute potential of the various ions. Before setting up any theoretical formula, it is necessary to get clearly in mind just in what the difference in potential between the protoplasm and the salt consists.

d) *Derivation of a theoretical formula expressing the relationship between minimum fatal dose and the available potential energy of salt solutions.*—We have now found out how to measure the potential of the potential energy of the salt solution; it is necessary that we discover also how the potential of the potential energy of the protoplasmic system is to be determined, since our formula involves both these factors, the available potential energy being the difference between the potentials of the salt and the protoplasm.

The protoplasmic system is made up of masses of proteid matter in equilibrium apparently with particles of the same proteid in solution. It may be regarded as a two-phase colloidal system. We may assume, in the light of the investigations of the past five years, that changes in the protoplasmic activity are due, in part at least, to changes in the state of these colloidal particles and masses, and that the salts are affecting vital processes in part by producing such changes. What we have to compare, then, in the first instance, is the potential of the energy of the protoplasmic colloids with the potential of the potential energy of the ions of the salt solution. We have, therefore, to get a clear idea of the relationship of salts to the precipitation and solution of colloids.

Since the protoplasmic colloids are for the most part composed of albumin in combination with other radicles, it is to the albuminous or proteid colloids to which attention may first be directed. The work of Kossel and Fischer has cleared up the structure of the albumin molecule, which has been shown to be a polymer of amino acids. As a result, albumin or proteid is shown to be both acid and basic; that is, it is capable of uniting with metals to form true salts, and also with acids through the amino group.

If common egg albumin is dissolved in alkaline solution, it exists as sodium albuminate; if it is dissolved in hydrochloric acid, it exists as albumin chloride. Sodium albuminate dissociates electrolytically, owing to the high dissociating power of sodium (that is, its high solution tension), and Na^+ and albumin ions are formed. Similarly, owing to the high dissociating power of the chlorine, albumin chloride dissociates into albumin and Cl^- ions. This dissociation results in giving the albumin—that is, the colloidal particle—a positive or negative electric charge. It is possible to change the sign of the charge on the albumin particles by making an alkaline solution sufficiently acid. This is brought about in the following way: By adding acid to the alkaline albumin the highly dissociated sodium compound is replaced by the slightly dissociated hydrogen compound. The result of this is that the charge on the colloid is neutralized, undissociated albumin is formed, and if the concentration of the albumin is sufficiently great, precipitation will occur. If one continues to add

acid, the albumin combines with the hydrochloric acid and goes over into the chloride. This at once dissociates the chlorine ion as a negative ion, and the albumin becomes the cation. It will, however, be clear that, although in this case the albumin is mainly electropositive, yet it is an acid, and the ionization of its hydrogen is not completely suppressed, although it is reduced to a very small amount. This means that here and there are albumin particles which are at one spot electronegative, since they dissociate hydrogen, and in another spot electropositive, since they dissociate chlorine. We have some amphoteric or twin ion colloids, in other words. The evidence of the existence of such ions will be taken up on p. 104.

I make this explanation at such length because it does not appear to be clear to many that the albumin colloids owe their charges to processes of ionization, just as any salts owe their charges to these processes. Thus several writers have assumed that the charge was owing to the salt introduced. The ion of the salt introduced which moved fastest was supposed to bury itself in the colloid particle, and thus give its charge to it. This hypothesis is quite unfounded and undoubtedly erroneous.

The proteids, therefore, in protoplasm exist as salts, and dissociate sodium or other metallic ions, and chlorine and other anions, and the colloids thus become charged. Some proteids here and there dissociate both positive and negative ions. The colloids in protoplasm are undoubtedly in the condition of a saturated solution, and we have an equilibrium between dissociated and undissociated colloids, in solution, and undissociated and dissociated precipitated colloids.* It has been shown, furthermore, that the state of solution and the fineness of subdivision of the colloidal particles depend on the number of free electrical charges on their surfaces. The greater the number of similar charges, the greater the solubility of the colloid.

In determining the potential of the potential energy of the colloid, we have, then, just the same factors to consider as in the salts, since the colloids are salts. The potential of the energy content of the colloid solution must be determined by the ionic potentials of the ions into which it dissociates, for example, the potentials of sodium and albumin.

*As a possible example of a dissociated insoluble colloid, fibrin which has been in acid may be mentioned. This fibrin dissociates hydrogen, but the hydrogen ion is unable to move away from the fibrin. The condition is very similar to the double layer at the surface of an electrode.

In studying the action of any salt on an albumin solution, the real question to be answered is: What will be the result upon the solubility and state of the colloid of replacing the ion already in combination with the proteid by some other ion containing a different amount of potential energy? We have to know, therefore, before we can answer the question of the action of any salt on a colloid, what the ion is which is already in combination with it. This is a very important point, which is frequently overlooked in studying the action of salts.

To get a clear idea of what happens when a salt is added to an albumin solution, let us consider first, the condition of affairs in the sodium albumin solution in which the proteid exists as $\text{Na} + \text{albuminate}$. As the colloid stands in a saturated solution, it is in a condition of equilibrium. The sodium ion has separated a certain distance from the albumin ion. The distance it moves depends, no doubt, on several factors, but the most important will probably be its tendency to go into solution—i. e., its solution tension.* The positive ion of sodium repels a negative charge with a power equal to 2.54 volts. What the negative solution tension of the albumin ion is, unfortunately is unknown. The effect of the positive charge on the sodium will be, of course, to neutralize the negative charge on the albumin, but, owing to the fact that the sodium repels the negative charge and holds its positive charge so firmly, it is unable to neutralize it, and the colloid remains in solution. We have, in other words, an equilibrium between dissociated and undissociated sodium albuminate.

The question, then, to be solved is this: What effect will it have on the solubility of the colloid if we replace the sodium ion by another ion containing a different quantity of potential energy, i. e., having a different ionic potential? One of two results may be anticipated: either the dissociation will increase and the colloid go more completely into solution, or it will diminish and the colloid be more or less completely precipitated.

If we introduce an ion of higher potential than sodium, evidently the state of equilibrium can no longer be the same. Energy will pass from the positive ion to the albumin, and will in some degree hold or neutralize its negative charge. We may imagine that the new

*Many facts indicate that one of the most important factors in determining the ionization of salts is the ionic potential of its ions. For example, compare the ionization constants of the iodide, chloride, and bromide of mercury or silver; or compare the ionization of silver and sodium nitrates.

ion no longer can move so far from the albumin, and in consequence more nearly neutralizes its charge. The result is that the surface of the colloidal particles will be reduced, the surface tension will be increased and the colloid will be less stable. In another way of putting it, the dissociation is somewhat reduced and consequently some of the colloid tends to precipitate. If, in fact, the ion used to supplant the sodium has a sufficiently high potential, it will practically not leave the colloid at all, the dissociation will be greatly reduced, the negative charges almost neutralized, and precipitation will occur. If the ionic potential of the introduced ion is still higher, it may oxidize the colloid; i. e., an actual exchange of charges will take place between the albumin and the ion.

If, however, an ion of lower potential is introduced in place of the sodium, the reverse of these processes will take place. Ionization will be increased, the negative charge will be freer, and the solubility of the colloid will be greater. This will be the case if potassium is substituted for the sodium, provided that potassium has a lower ionic potential than sodium, as is generally assumed, and that no other factors come into play.

It is, therefore, clear that the effect of any salt upon a saturated colloidal solution of albumin in which the albumin is electronegative will depend chiefly upon what ion is in combination with the colloid when the salt is introduced.

The quantitative differences in the effects of different salts must depend upon the differences in the ionic potentials of the ion in combination with the proteid and that substituted for it.

So far, we have considered only the rôle of the positive ion. That of the negative ion is also of importance, but somewhat more difficult to picture to ourselves. We may, however, look at it in this way. The different negative ions introduced have different tendencies to deposit on the colloid, and give up their negative charges to it. This tendency is measured by the ionic potential of the ion. If the negative ion does deposit, it will tend to increase the negative charge on the colloid, and hence to dissolve it. The higher the ionic potential of the anion introduced, the greater must be its dissolving action on the colloid, since the greater will be its tendency to give its negative charge to the albumin. If the ionic potential of this ion is

lower than the albumin, it will have an opposite or precipitating action, since then the colloid will tend to give up its charge to it.

These conclusions are confirmed by my results on sodium albuminate, and those of Osborne and Harris on edestin.

From these general considerations the conclusion may be drawn that the precipitating action of the salt on the colloid will be proportional to the difference between the ionic potential of the positive ion already combined with the colloid and that substituted for it; and that the dissolving action of the anion will be proportional to the difference in potential of the anion of the colloid and that we introduce; or

$$\text{Precipitating action} = E_{c \text{ salt}} - E_{c \text{ colloid}}.$$

$$\text{Dissolving action} = E_{a \text{ salt}} - E_{a \text{ colloid}}.$$

In this formula $E_{c \text{ salt}}$ is the ionic potential of the cation of the salt introduced, and $E_{c \text{ colloid}}$ that of the cation of the colloid. $E_{a \text{ salt}}$ and $E_{a \text{ colloid}}$ are the values for the anions.

Since these two actions are mutually antagonistic, the actual action of the salt will be equal to the difference between them, or

$$\text{Actual action} = \text{precipitating} - \text{dissolving action}$$

$$= E_{c \text{ salt}}^i - E_{c \text{ colloid}}^{ii} - E_{a \text{ salt}}^{iii} + E_{a \text{ colloid}}^{iv}$$

$$= (E^i - E^{iii}) - (E^{ii} - E^{iv}).$$

If the result is positive, the salt should precipitate; if it is negative, it should dissolve the colloid. If it is zero, the salt should not affect the colloid except by mass action or by action on the water. In other words; *the actual action of any salt on a colloid in solution will be proportional to the difference between the ionic potentials of the ions of the salt, minus the difference in ionic potentials of the ions of the colloid.*

Let it be assumed that the logarithm of the dilution of the least precipitating concentration, or the logarithms of the dilution of solutions of equivalent dissolving power, are proportional to the actual action of the salt.¹ This gives the following equation:

$$\log V = K[(E^i - E^{iii}) - (E^{ii} - E^{iv})] + \text{const.} \quad (2)$$

Comparing two salts with the same sign of action, i. e., dissolving or precipitating.

$$\log V_1 = K[(E_1^i - E_1^{iii}) - (E^{ii} - E^{iv})] + \text{const.}$$

$$\log V_2 = K[(E_2^i - E_2^{iii}) - (E^{ii} - E^{iv})] + \text{const.}$$

$$\log \frac{V_1}{V_2} = K[(E_1^i - E_1^{iii}) - (E_2^i - E_2^{iii})]. \quad (3)$$

¹ See Fig. I, p. 96.

That is, *the logarithm of the ratio between equivalent precipitating concentrations of two salts, divided by the difference between the differences of potentials of the ions of the two salts ought to give a constant.**

We thus have for the first time a formula for application to protoplasm which states clearly at the outset that the effect of any salt solution on the protoplasm will depend upon what ions are already in combination with the protoplasm. In other words, if we supplant most of the ions in any cell by sodium, and then apply calcium chloride, the effect will be different from that obtained if calcium chloride is applied before the sodium chloride. Furthermore, the same salt will act differently on different cells, if only those cells have different ions in them. Both of these necessary conclusions of the theory have been established by observation. To make this perfectly clear, the difference in potential between the protoplasm and the salt solution which we started to measure is the difference in potential between the systems ionized colloid—ionized salt.

However, this formula cannot be applied directly as it stands to protoplasm as a whole, because it only applies to colloidal solutions in which the colloids are all of one sign. In protoplasm, however, it is certain that we have colloids of both signs and very probably amphoteric colloids; i. e., colloids which are both positive and negative at different parts of the molecule.† We probably have, in other

* This formula may, I think, be substituted with advantage for that of the tension coefficient. It is in reality the numerator of the tension coefficient.

† A number of facts speak for the presence of such twin ions in colloidal albumin solutions and in protoplasm. For example, if egg albumin is dialyzed nearly free from salts, and then coagulated so as to form a weakly alkaline colloidal solution of albumin, and if this solution is then made acid, it is well known that the albumin becomes predominantly electropositive. That is shown by the colloid migrating slowly to the cathode in an electric field, and also by the combining power of the albumin, since it now combines readily with picric and other acids to form albumin picrate, tannate, and so on. Nevertheless, if the solution is not too acid, it will combine still with the metals in some measure. This is undoubtedly due to the composition and character of the albumin. The alkali albumin first obtained by heating is a salt. When the albumin has acid added to it, there is formed, in the first instance, the free acid of the albumin, which is not much dissociated. In addition, the acid is added to the amido-group, and in an excess of acid hydrolytic decomposition being greatly reduced, the dissociation takes place so as to make the albumin predominantly electropositive. However, the ionization of the acid is not entirely prevented, although it is greatly reduced, so that in some places we must have some hydrogen ions being formed leaving the albumin electronegative at certain places. It is probably this small percentage of hydrogen ions which can still be replaced by the metals. I found that, as a matter of fact, the heavy metals mercury and copper, although they would not in themselves cause a precipitate if the solution were sufficiently acid, yet they rendered the albumin far more easily precipitated than it was before. This is to be anticipated on our view that the colloidal particles are in some places negative and in other places positive. Not only does this appear to be the case for albumin, but in protoplasm there is also reason for believing that both ions of the salt are actually bound by the protoplasm, and especially in *Fundulus* eggs. It will be remembered that Du Bois Raymond long ago assumed that such polarized particles might exist in proto-

words, ions like that in Fig. 2. As a matter of fact, if we try to apply this formula to the results on toxicity, a discrepancy between the response of protoplasm and colloidal albumin to salts is at once noticed. I have already called attention to this discrepancy. The discrepancy is this: While in the colloidal solutions the opposite action of the ions, dissolving and precipitating, is clearly apparent,

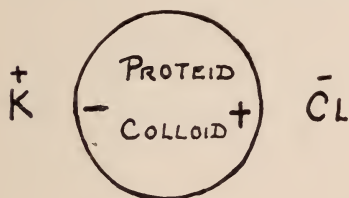


FIG. 2. Illustrating a colloidal twin ion dissociating at one place K, and at another Cl, leaving the colloid both negative and positive at different places.

and that opposite action is proportional to the ionic potentials of the anion and cation respectively, for protoplasm in general and for the ferment studied by McGuigan a different relationship is seen in that, instead of the positive ion counteracting by its energy content the negative ion, as it ought to do on the theory developed, a summation

of effects is noticed. The iodides of the metals, instead of being less poisonous than the chlorides, as they should be, are more poisonous. The explanation of these facts is to be sought on the basis of the differently charged colloids present.

If these amphoteric colloidal particles exist in protoplasm, or if we are dealing in protoplasm with a mixture of both negative and positive colloids, each ion will tend to precipitate. In the twin ions, if potassium is replaced by an ion of greater potential energy, the proteid will tend to be precipitated, since the negative part will be partially neutralized; and similarly if chlorine is replaced by an anion of greater potential. Both ions, therefore, will exert an action in the same direction, and there will be a summation of action in this case instead of a difference. The formula for toxicity would become:

$$\begin{aligned} \text{Total action} &= (E_{\text{cation salt}} - E_{\text{cation colloid}}) + (E_{\text{anion salt}} - E_{\text{anion colloid}}) \\ &= (E_{\text{cation salt}} + E_{\text{anion salt}}) - (E_{\text{cation colloid}} + E_{\text{anion colloid}}) ; \end{aligned}$$

or, writing E'_c and E'_a for the ionic potentials of the anion and cation of the salt introduced, and E_c and E_a for the ionic potentials of the ions bound to the colloid,

$$\begin{aligned} \log V^i &= K[(E_c^i + E_a^i) - (E_c + E_a)] + C \\ \log V^{ii} &= K[(E_c^{ii} + E_a^{ii}) - (E_c + E_a)] + C \end{aligned} \quad (4)$$

$$\log \frac{V^i}{V^{ii}} = K(E_c^i + E_a^i - E_c^{ii} - E_a^{ii}) \quad (5)$$

$$\frac{1}{E_c^i + E_a^i - E_c^{ii} - E_a^{ii}} \log \frac{V^i}{V^{ii}} = K \quad (6)$$

That is, the logarithm of the ratios of the dilution of the minimum fatal doses of two salts, divided by the difference of the sums of the ionic potentials of the two salts, is a constant.

This formula is very similar to that derived by me empirically from a study of the dilutions of the minimum fatal doses of salts toward the eggs of *Fundulus heteroclitus*. The empirical formula was

$$V_a = \frac{V_o}{\frac{E_a - E_o}{20.15 + 0.02 E_a}}.$$

In this formula E_a and E_o were the decomposition tensions of the salts.

If we take instead of 2 the base of the Napierian logarithms 2.718, and instead of $\frac{1}{0.15 + 0.02 E_a}$ we write K , this goes over into the form

$$V_a = V_o 2.718^{-K(E_a - E_o)}.$$

Taking natural logarithms

$$\log V_a = \log V_o - K(E_a - E_o)$$

$$\log \frac{V_a}{V_o} = -K(E_a - E_o).$$

This, in other words, is the same expression as that already derived, using the decomposition tension; i. e., the sum of the solution tensions of the ions, in place of the sum of the ionic potentials.

The formula may also be derived in another way. If V is the dilution of the minimum fatal dose, and if we let X represent the difference between the sum of the ionic potentials of protoplasmic ions and salt ions, obviously from the form of the curve $\frac{dv}{dx}$ varies with its position on the curve, that is with V

$$\frac{dv}{dx} = KV,$$

$$\therefore \log V = KX + C = K(E_c^i + E_a^i - E_c - E_a) + C.$$

An application of this formula to the results of McGuigan and myself give the following values for K . In each case it is assumed

that the original ions in combination with the colloids are K and Cl .*

TABLE 5.

Salts Compared	$\frac{1}{E'_c + E'_a - E''_c - E''_a}$	$\log \frac{V_1}{V_2}$	K
$CuCl_2 - MnCl_2$	(1.403) —I	3.1249	2.23
$HgCl_2 - MnCl_2$	(1.816) —I	3.6812	2.03
$NiCl_2 - MnCl_2$	(0.848) —I	2.163	2.55
$AgNO_3 - HCl$	(0.8850) —I	2.0044	2.26
$CuCl_2 - CdCl_2$	(0.7567) —I	1.7655	2.33
$NiCl_2 - CdCl_2$	(0.201) —I	0.8035	3.997
$CdCl_2 - MgCl_2$	(1.072) —I	2.1553	2.010
$HgCl_2 - MgCl_2$	(2.241) —I	4.477	1.998
$CuCl_2 - MgCl_2$	(1.828) —I	3.9208	2.145
$ZnCl_2 - MnCl_2$	(0.302) —I	1.0430	3.454
$ZnCl_2 - NiCl_2$	(0.546) —I	1.1201	2.051
$HgCl_2 - CoCl_2$	(0.972) —I	2.4771	2.548
$HgCl_2 - CuCl_2$	(0.4121) —I	0.5563	1.35
$CoCl_2 - MnCl_2$	(0.843) —I	1.2041	1.428

Mean value of K 2.23

The values of K (Table 5) are on the whole fairly constant for the great majority of the salts compared. The variations from the mean of 2.2 are due almost entirely to the fact that mercury is not so poisonous as it should be, and that cobalt is a good deal less toxic than the theory demands, while nickel is a little more toxic. The explanation of these variations is no doubt to be found in part in the dissociation. I have assumed throughout that the dissociation is complete. This has been done for the sake of simplicity. It is, however, certain that the dissociation of mercury chloride even in these dilutions is far from

*Some modification or explanation is necessary of the conclusion of a former paper that oppositely charged ions must have of necessity an opposite action. This is in one sense true. That is, the positive ion in combination with the proteid, if the latter is electronegative, must constantly be neutralizing the negative charge and producing undissociated albumin. It may be stated in this sense that the positive ion always tends to precipitate an electronegative albumin. It happens, however, that the power of neutralizing the charges of the colloid—that is, of reducing ionization—varies greatly in different cations, being greatest in those of high ionic potential, and least in those of low. If, therefore, we have, as we do have in a protoplasmic system, colloids in a state of equilibrium with ions already present, the particular direction of the change in state of that equilibrium produced by the substitution of new positive or negative ions for those already present will depend on the relative potentials of the ions present and those introduced in their places. The actual effect observed, therefore, of replacing an ion of high potential with that of a low, will be the direct opposite of that produced by replacing the ion with an ion of still higher potential, and in this case there will appear to be an antitoxic or antagonistic action between two ions of the same character of charge. In an earlier discussion of this matter I neglected to take into account the great importance of the ions present in protoplasm. For example, suppose the ions in the protoplasmic system to be mainly sodium; and let us suppose that potassium has a lower potential than sodium, while calcium has a higher potential. If one substitute calcium for the sodium, the result will be to precipitate in part the electronegative colloids in the protoplasm. If, however, potassium be substituted for the sodium, the result will be to dissolve still further the colloids. In this case potassium and calcium will appear to exert an antagonistic action toward each other. If, however, the ions already in the protoplasm are of higher potential than calcium, then both potassium and calcium will produce the same kind of an action on the protoplasmic colloids. The results obtained by Loeb, Loeb and Giess, Miss Moore and myself on toxic and antitoxic action of salts thus have a very simple explanation.

complete. If we assume it to be only 50 per cent, it would bring the mercury into its proper position. Similarly with cadmium, which is a trifle too low in its toxicity, this dissociation is certainly incomplete. As regards cobaltous chloride, which is noticeably below what it should be, I have no explanation to offer except to point out that it occupies the same exceptional position toward some other forms of protoplasm. Possibly its power of forming double compounds with ammonia and its derivatives may have something to do with its anomalous behavior.

The constancy of K must be regarded, I think, with the exceptions just mentioned, as satisfactory, when it is remembered that the method of determining the minimum fatal dose—that is, by dilution—does not permit of very accurate figures.

C evaluated from these figures was—4.111. (See Formula 4.)

TABLE 6.
RESULTS ON *Fundulus* EGGS.

Salts Compared	$(E'_c + E'_a - E''_c - E''_a)$	$\log \frac{V_1}{V_2}$	K
CuCl ₂ —MnCl ₂	(1.403)	3.574	2.547
HgCl ₂ —MnCl ₂	1.816	4.097	2.260
HgCl ₂ —CuCl ₂	0.412	0.5220	1.269
NiCl ₂ —MnCl ₂	0.848	2.0969	2.473
CoCl ₂ —MnCl ₂	0.843	1.796	2.131
HgCl ₂ —MgCl ₂	2.241	4.398	1.964
CuCl ₂ —MgCl ₂	1.828	3.875	2.120
CuCl ₂ —NiCl ₂	0.556	1.477	2.655
HgCl ₂ —CoCl ₂	0.972	2.301	2.367

Mean value $K = 2.20$

TABLE 7.
COMPARISON OF ZN WITH ALL OTHER METALS.

Salts Compared	$(E'_c + E'_a - E''_c - E''_a)$	$\log \frac{V_1}{V_2}$	K
ZnCl ₂ —MgCl ₂	0.726	2.602	3.581
ZnCl ₂ —MnCl ₂	0.302	2.301	7.610
CdCl ₂ —ZnCl ₂	0.345	1.194	3.461
CoCl ₂ —ZnCl ₂	0.541	—0.5051	—0.934
NiCl ₂ —ZnCl ₂	0.546	—0.2041	—0.374
PbCl ₂ —ZnCl ₂	0.613	0.795	1.296
CuCl ₂ —ZnCl ₂	1.102	1.273	1.182
HgCl ₂ —ZnCl ₂	1.514	1.7959	1.186
AgNO ₃ —ZnCl ₂	1.597	2.097	1.314

Mean $K = 2.03$

The results obtained upon *Fundulus* (Tables 6 and 7) do not give quite such constant values as those of McGuigan, and indeed with so complex a system this was not to be expected. However, the

average value of K in these results is almost exactly the same as that of McGuigan; i. e., about 2.2. The exceptions in these results are, with the exception of cadmium, the same as those recorded by McGuigan, cobalt being too little toxic and zinc too toxic. I have determined K for zinc chloride in comparison with all other metal chlorides, expecting that, while the ratio would be too low for all other metals above it in the scale of ionic potentials, it would be too high for all metals below it, and these two errors should neutralize each other, provided the metals were about equally distributed above and below zinc. The result (Table 7) gave for the mean K 2.03, which is a little low, but fairly close to the mean 2.2 already obtained.

I have also determined (Table 8) the fatal dose just sufficient to stop swimming in two minutes in rapidly swimming cultures of *Volvox globator*. Four metals were investigated—i. e., silver, cadmium, manganese, and magnesium. K was assumed to be 2.2, the same as that for *Fundulus* and diastase, and the constant C was calculated.

The result was as follows:

TABLE 8.

Salt	$\log V$	C
AgNO ₃	5.477	-3.5
CdCl ₂	2.699	-3.6
MnCl ₂	1.699	-3.11
MgCl ₂	0.699	-3.13

Mean—3.33

The constant C is thus found as constant, as could be expected from the methods used and the variability of the cultures.

Undoubtedly the most consistent and accurate results thus far obtained are those of McGuigan upon the minimum fatal dose of salts for the diastatic ferment. These results are plotted in Fig. 3. I have used only those results which were obtained with the metals Mg, Mn, Zn, Cd, Co, Ni, H, Cu, Hg, and Ag, for the reason that the solution tension, and hence the ionic potential, of all metals above magnesium—i. e., Ca, Sr, Ba, Na, K, Li, and Cs—are still so very uncertain as to make the comparison of ionic potential and fatal doses of little value. In Fig. 3 the line AB represents the formula.

$$\log V = C + K(E_{\text{cation salt}} - E_{\text{cation colloid}}) + (E_{\text{anion salt}} - E_{\text{anion colloid}}) .$$

The ordinates represent the logarithms (common) of the dilution (V) of the minimum fatal dose; the abscissæ represent the differences between the ionic potential of the cation of the colloid, which is assumed to be potassium at 2.9 volts and the ionic potential of the various

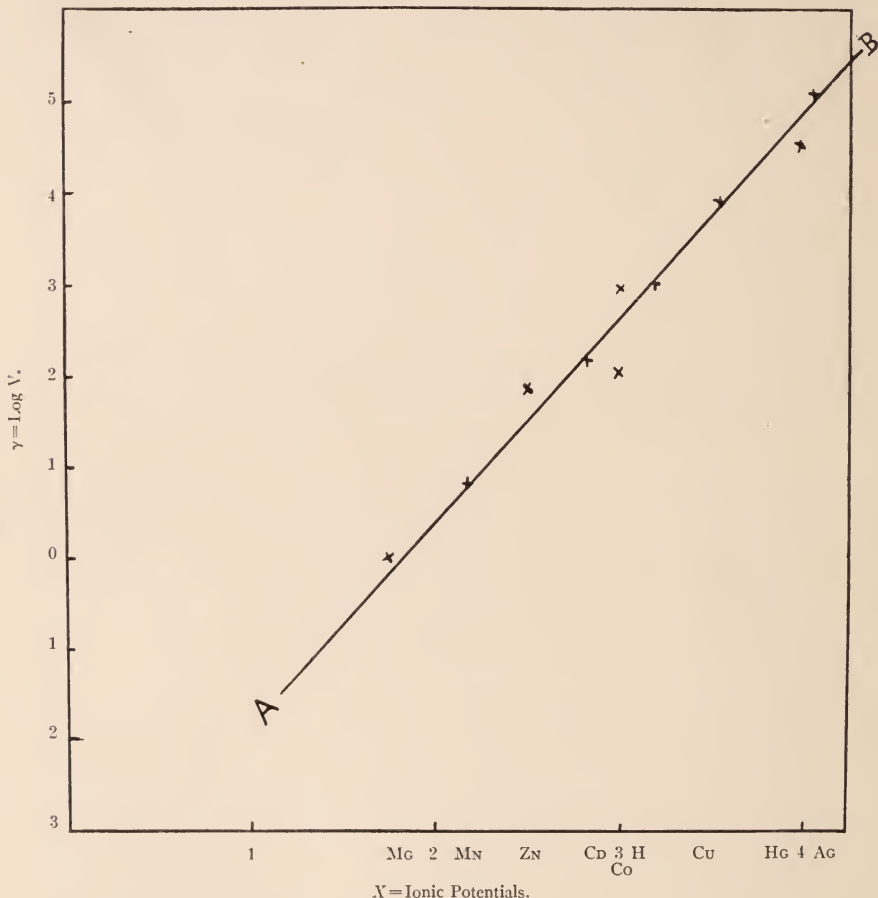


FIG. 3. Plat of results obtained with diastase. Ordinates are the logarithms of the dilution of the minimum fatal dose. Abscissæ represent the difference between the ionic potentials of K. Cl and potentials of ions of toxic salts.

metals. The line makes an angle with the X -axis, the tangent of which is 2.23, and it cuts the Y -axis at -4.11 (or C).

The remarkable closeness with which the various metals approximate to this line will be apparent.

If now we turn to my results on *Fundulus* (Fig. 4), two things are at once clear from an inspection of Table 6 and Fig. 4. The first result is that the gradient or slope of the line which represents the relation between toxicity for *Fundulus* eggs and the ionic potential is

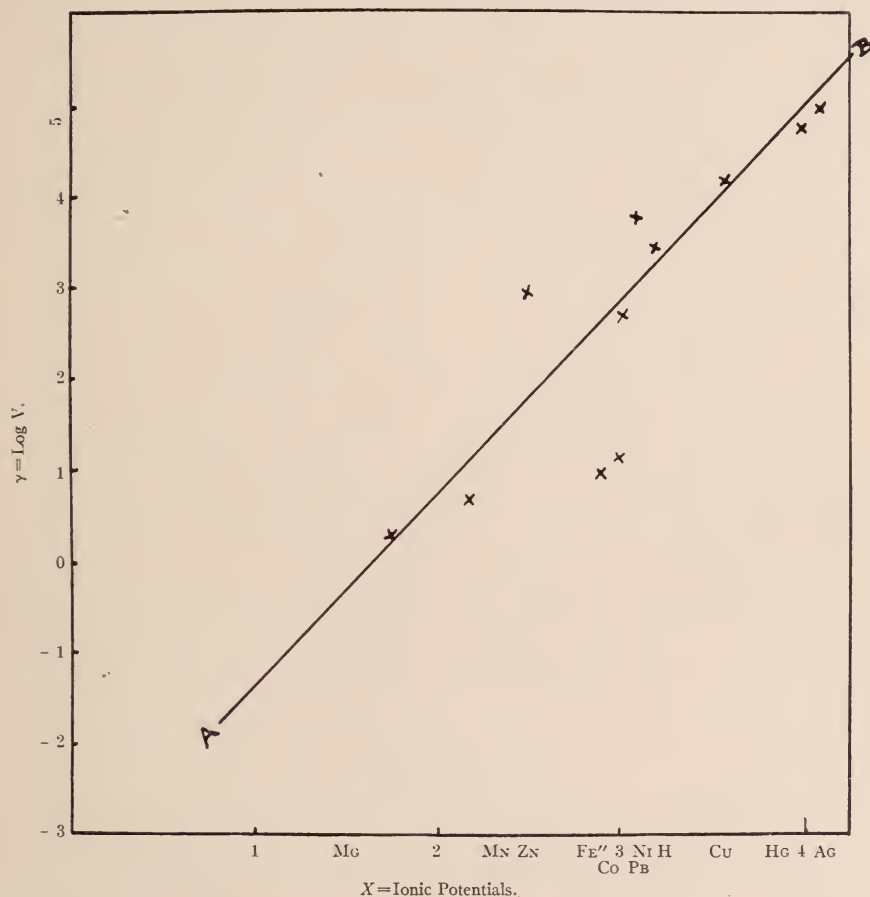


FIG. 4. Plot of results obtained with eggs of *Fundulus heteroclitus*. Ordinates and abscissæ as in 3.

exactly the same as that found for diastase. This must be regarded as confirmatory evidence of some value of the probable correctness of our attempt to work out a numerical relationship between toxicity and ionic potential. The second result which is very clear is that there is in the case of *Fundulus* greater variations than in

the case of the ferment. This is, of course, to be expected since in the egg we are dealing with a vastly more complicated system than in the ferment. We have not only a variety of ferments and substances which might be differently affected by the salts, but the eggs are in addition separated from the water in which the salts are by membranes which are known to be variously permeable to different salts. The fact that the results show so good an agreement with the computed values is hence the more satisfactory. As regards the exceptions, it will be observed by comparing Figs. 3 and 4 that they are in general the same in each, only the deviations are greater for the egg. Thus zinc, which toward diastase was somewhat more poisonous than it should be, toward the eggs is very much more toxic. Cobalt, which is too little toxic toward diastase, shows the same relationship toward the eggs; the same is true of mercury. On the other hand, certain very interesting special exceptions occur. Thus cadmium, which toward diastase occupies almost exactly its theoretical position, is toward *Fundulus heteroclitus* eggs extremely toxic. It is, in fact, so toxic and so far out as to show that there is some specific and special reason for its aberrance. I have accordingly disregarded it. On the other hand, lead, which was for some special reason far out of place toward diastase, is here almost where it should be.

As regards the toxicity of the metals sodium, potassium, and lithium it will be noticed that they are relatively more toxic toward *Fundulus* than toward the diastase. The reason for this may possibly be that the strong solutions are in themselves harmful by their osmotic action on the cells.

I have also incorporated the results of a few observations made upon the rapidly swimming culture of *Volvox globator* (Fig. 5). I determined the concentration just sufficient to stop swimming within two minutes. The computation of the constant a , from the results gives a very satisfactory agreement.

e) *Other results on toxicity.*—The results of Caldwell on bromelin, the proteolytic ferment of the pineapple, I have been unable to bring to any satisfactory numerical agreement. But while Caldwell's results cannot be brought into quantitative relationship with McGuigan's and mine, the general trend of the results is plainly the same. The order of the toxicity of the metals is in nearly all cases as it should theoretic-

cally be, so far as he has tried those of which the solution tension is known. The same exceptions are also apparent. Thus cobalt is not sufficiently toxic, and zinc is too toxic for the rule. Lead is about where it belongs, but in this case barium is the marked and peculiar

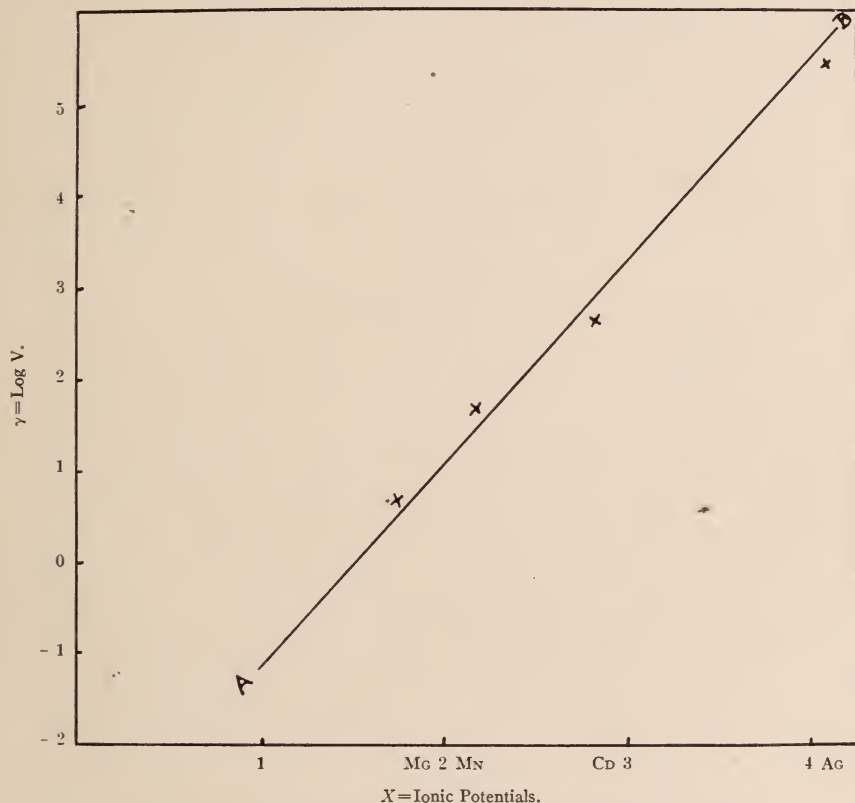


FIG. 5. Plot of results obtained with *Volvox*. Abscissæ and ordinates as in Fig. 3.

exception, in place of the lead toward diastase, and cadmium toward the *Fundulus* egg.

The results of Heald (Table 1) so far they have been obtained with the salts we are examining, show much the same order of efficiency. Thus for *Pisum sativum* the order of toxicity is: Ag, Hg, Cu, Ni, Co, and H; while for *Zea mais* it is Ag, Cu, Hg, Ni, Co, and H. These results are not of a sufficiently definite character, based as they are on the growth of roots, to enable very accurate quan-

titative comparisons. It will be noticed that nickel is more poisonous than cobalt for *Pisum sativum*, and far more poisonous than cobalt for *Zea mais*. Both of these roots appear less sensitive to acids than to the metals, the hydrogen ion being less toxic than nickel. With *Lupinus*, while the general order is the same as that observed elsewhere, cadmium is here more poisonous than copper—just the exception noted for *Fundulus*.

The results of Clark and Stevens upon mold spores are also for the purposes of quantitative comparisons unsatisfactory. These spores appear to be surrounded by such membranes, or to have so great a resistance, as to make the interpretation of results very doubtful, although there can be no doubt that the general trend of the results is the same as that already noted.

True and Kahlenberg's results (Table 1) are more satisfactory, but, owing to the fact that these authors did not study many of the metals, and also did not pretend to fix the fatal point with accuracy, their results are not satisfactory for quantitative treatment. True's results upon the toxicity of the salts of the various acids are unfortunately unavailable for our purposes, owing to the uncertainty of the ionic potential of these anions.

f) *The solubility of globulin in salt solutions*.—In a previous paper¹ I showed that the solubility of sodium albuminate (egg albumin in alkaline solution) in different salt solutions was determined by the tension-coefficient of the salt. By the tension-coefficient was meant the difference between the solution tensions of the ions divided by their sum. The numerator of this fraction should be the ionic potentials instead of the solution tensions. I showed that in an alkaline solution the solubility was greater in sodium iodide than in the bromide or chloride, and that when the ionic potential of the cation surpassed a certain figure the salts precipitated the albumin. Table II³ shows the relationship (qualitative) between the ionic potentials of salts and their power of dissolving or precipitating such albumin, both the unboiled and the boiled. It will be seen by an inspection of the table that the ionic potential arranges the salts in the order of their action on the albumin.

Osborne and Harris² have estimated quantitatively the solvent

¹ MATHEWS, *Amer. Jour. Physiol.*, 1905, 14, p. 204.

² OSBORNE AND HARRIS, *ibid.*, 1905, p. 151.

³ MATHEWS, *loc. cit.*, p. 211.

power of many salts for the globulin of the hemp seed edestin. I append their results here to show how far they agree with the theoretical deductions which were worked out on p. 102. In Table 9 $E_c - E_a$ represents the difference between the ionic potentials of the salts;

TABLE 9.

Salt	$E_c - E_a$	c.c. to Dissolve 1 gr.	K
KI.....	-2.123	5.7	
KBr.....	-1.65	10.	.51
KCl.....	-1.226	15.1	.44
NaI.....	-1.743	5.7	
NaBr.....	-1.27	9.3	.44
NaCl.....	-0.846	12.8	.33
LiBr.....	-1.10	12.1	
LiCl.....	-0.646	19.1	.44

the third column represents the number of c.c. of a normal solution of the salts which will just dissolve one gram of edestin. I have taken these figures from the chart given by Osborne and Harris. They are only approximate. The theory requires that the more negative the difference $E_c - E_a$, the smaller the amount of salt necessary to dissolve a given amount of the edestin. It will be seen that this relationship holds very well if we compare the iodides, chlorides, and bromides of sodium, lithium, and potassium respectively. Unfortunately, the great uncertainty of the solution tensions and ionic potentials of sodium, potassium, and lithium, prevent quantitative comparisons between the different metals. In the fourth column under K, I have computed the constant by the formula on p. 102, using instead of the logarithm of the dissolution the logarithm of the ratios of number of c.c. of the different solutions necessary to dissolve one gram. The figures are placed between the salts compared. The formula used was:

$$\frac{1}{(E_{c_1} - E_{a_1}) - (E_{c_2} - E_{a_2})} \log \frac{c.c._1}{c.c._2} = K.$$

Osborne and Harris draw the conclusion that the solubility is independent of the nature of the base, but I think their figures speak for themselves. The differences between potassium, lithium, and sodium are small, to be sure, but nevertheless apparent. The importance of the base becomes obvious so soon as any other salts are examined in which the base has a higher ionic potential than

these. Then it is seen that whether the salt dissolves the edestin or converts it into a curdy mass depends mainly on the base. The reason why so slight differences exist in the solvent power of the cations, sodium, potassium, lithium, barium, calcium, and magnesium, is shown by an examination of their ionic potentials, which are very low and probably about the same in each. The solvent power of manganese and ferrous chlorides is low.

Cu, Cd, Cr, Co, Fe''' , Pb, Hg, Cu, Al, Zn chlorides and nitrates all fail to dissolve.

The solvent powers of the anions arrange themselves in descending order as follows: CrO_4 , SO_3 , S_2O_3 , I, Br, Cl, SO_4 , which is almost certainly the descending order of the ionic potential of these ions. The fact that these bivalent ions of high ionic potential dissolve, instead of precipitating, the colloid, and that the valence of the anion is unimportant, is, in my opinion, good evidence that the edestin in such solutions is electronegative and not electropositive; for, as has been shown by Hardy and many others, the valence of the ion of the same sign as the colloid is immaterial, but toward a colloid of opposite sign it is very important.

The peculiarity of the dissolving action of the heavy metal acetates¹ also receives a possible explanation. In these solutions which dissociate acetic acid the edestin becomes electropositive. Consequently it is no longer precipitated by the positive ions, but receives energy from them, and is rendered more positive, and hence more soluble, than it was before. The cause of the failure of the chlorides to dissolve the edestin in acid solution would be that given by Osborne, that in such solutions, where there are many hydrogen ions, it is changed into edestan as an insoluble product.

I append here also a summary of the results of Pauli² illustrating the same facts, showing the parallelism between the solvent or precipitating power of the anion upon albumin and its potential energy content. The parallelism is certainly unmistakable.

Order of precipitation	$\text{SCN} > \text{I} > \text{Br} > \text{NO}_3 > \text{Cl} > \text{C}_2\text{H}_3\text{O}_2$
Ionic potential	$83(?) - .79 - 1.27 - 1.694$

g) *The phenomena of stimulation of cells.*—I found that the salts ranged themselves very simply in the order of their energy content,

¹ OSBORNE AND HARRIS, *loc. cit.*, p. 165.

² PAULI, *Hofmeister's Beiträge*, 1905, 6, p. 249.

so far as their action, stimulation or depression, on the motor nerve was concerned. But it is clear from what has been said, that no such simple arrangement is to be anticipated in studying a complex system such as a cell undergoing rapid change. In such a system, all we observe from the action of the salt is a definite result, which implies a certain change in the system. This result may be a stimulation, such as a muscle contraction, or nerve impulse. Evidently the same result may be brought about in several different ways: either by direct action of the salt on the particular part of the system which undergoes change; or, indirectly, by the salt altering another part of the system, so as to produce or check the result. It is conceivable that the same salt may have a double action: by one action tending to produce the direct change of the response; and indirectly by action on another part of the system having as a result the setting-up a process which will check its own direct action.

Something of this last process obtains, I believe, in protoplasm generally, so that strict adherence to the law of ionic potential action is not to be expected; but a general adherence is to be expected, and the facts show it exists.

An interesting example of this stimulating action is seen in the extrusion of polar globules in *Chaetopterus* eggs. These eggs undergo the first processes of maturation before they are shed, but they do not extrude the polar globules until fertilized. The first polar spindle is formed and comes to rest in the equatorial plate-stage. Evidently in this egg there is not sufficient energy in the spindle to overcome the resistance offered by the surface tension or

TABLE 10.
MINIMUM CONCENTRATION OF VARIOUS SALTS CAUSING EXTRUSION OF POLAR GLOBULES IN CHAETOPTERUS.

Salt	Concentration
Na ₃ citrate	$\frac{7}{108} n$
Na ₂ SO ₄	$\frac{1}{5} n$
KI	$< \frac{5}{48} n$
KBr	$< \frac{1}{40} n$
KCl	$\frac{1}{12} n$
NaCl	$\frac{5}{82} n$
LiCl	$< \frac{1}{8} n$
CaCl ₂	$\frac{1}{16} n$
MgCl ₂	0 (?)
MnCl ₂	0
CdCl ₂	$< \frac{1}{1,600} n$
CuCl ₂	$< \frac{1}{24,506} n$

other factors, of the egg. Salts may cause extrusion either by increasing the energy in the egg, or by decreasing the surface tension.

Unfortunately the end-points were not determined accurately in many instances, but the figures (Table 10) suffice to show that the stimulation of this particular function was greatest in copper and cadmium, fell to nothing in manganese, was doubtful in magnesium, and then increased as salts having anions of higher ionic potential were used. In other words, if we begin with manganese or magnesium chlorides and pass to salts having a higher potential energy of either the anion or cation, this function is stimulated, and less of the salt must be used as the energy content increases.

GENERAL CONCLUSIONS.

The general conclusions of this investigation are:

1. The action of salts upon the protoplasmic system is due chiefly to the ions of the salt.

2. The particular result obtained—toxic, stimulation, or depression excited by any salt solution—is caused, in part at least, by the substitution of the ions in the protoplasmic system, and in large measure in combination with the protoplasmic colloids, by the ions of the salt solution used.

3. This substitution causes a disturbance of the equilibrium of the protoplasmic system, which, if sufficiently pronounced, leads to destruction.

4. *The power of different ions to upset the ordinary state of the protoplasmic system depends on the difference between the potential energy content of the ion which is replaced, and that which is introduced. This difference in potential energy content is determined by the difference in the intensity factor of the potential energy of the ions—i. e., by differences in the ionic potentials.*

5. It follows from 4 that the ions must arrange themselves in toxic power according to their available potential energy contents (ionic potentials). This was shown to be the case not only for toxic action, but also for stimulating and depressing action.

6. It was shown that a good numerical relation exists between the *available* potential energy of any salt and its minimum fatal dose, so that for simple systems the minimum fatal dose can be very

closely calculated from the available potential energy of the salt, if certain constants are known.

7. A method was found for computing the ionic potential of various ions from the solution tensions.

8. In the case of *Fundulus* eggs, which were particularly investigated from this point of view, the order is also consonant with the theory, but the quantitative relationships, while fairly good, are not so uniform as in the case of diastase.

The character of the action of any given salt solution on protoplasm must of necessity depend upon the character of the ions, metal and metalloid, already in combination with the protoplasm. This results as a necessary consequence of the theory, and it explains the fact that toward different cells, and toward the same cell after exposure to different salt solutions, the same salt solution may exert a different action, being at times stimulating, at other times depressing.

It has been shown that the physiological action of any salt solution is a function of the *available potential energy of its ions*. The action of the organic drugs will, I think, also be found to depend, in part at least, on the available potential energy of the dissociated particles of the drug.

STUDIES ON SIMPLE AND DIFFERENTIAL METHODS OF STAINING ENCAPSULATED PNEUMOCOCCI IN SMEAR AND SECTION.*

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IN the course of some experimental studies on pneumococcus infection, the technic of previous observers recommended for the staining of the encapsulated organisms was tested, and various new methods were devised in the hope of securing reliable procedures by which pneumococci may be differentially stained in cover-glass preparations and in sections of tissue.

My experience with these methods, old and new, it is the purpose of this paper to record.

PREVIOUS METHODS.

Simple staining.—In the simple† routine staining with aqueous-gentian-violet or carbol fuchsin, smears of pneumococcus exudates may show the organisms encapsulated; but this is extremely uncertain. Similarly, under favorable conditions many of the older special methods‡ devised for capsule staining often give excellent preparations, but the results vary, and are therefore unreliable when compared with those obtained with the simple, perfected technic used by recent observers. The most reliable and practical of all these methods in my experience are based wholly or in part on principles first adopted by Guarneri.

In 1888 Guarneri⁵ made determinations of the solubility of the pneumococcus capsule in acid, alkaline, and neutral salt solutions; and finally he obtained the proteid reaction with Millon's reagent, thus indicating an albuminous composition. On these determinations Guarneri devised a new method of staining the encapsulated organisms in exudates: smears fixed in the flame

*Received for publication February 19, 1906.

†Panc.^{1,2}

‡Friedländer,³ Ribbert,¹³ Roux,¹⁴ Muir,¹¹ MacConkey,⁹ Gordon,⁴ Kolle and Wasserman.⁸

were stained with analin-gentian-violet, then washed and differentiated in 2 per cent aqueous sodium chloride, rewashed very quickly in water, dried, and mounted in balsam. Similar methods have since been recommended. Thus, Welch¹⁶ adopted Guarneri's method of staining, but mounted the specimen in the salt solution, and suggested a preliminary treatment with glacial acetic acid on the ground that the capsule was composed of mucin.*

Buerger² also adopted Guarneri's method, but recommended a preliminary fixation of the capsule, first in a solution of chromic acid and bichloride of mercury, then in an alcoholic solution of iodine (U. S. P.). With Gram's method of staining this fixation is essential, but in the simple procedures the advantage of it is less apparent, for practically the same results are secured with Guarneri's less complicated method.

Hiss,⁶ however, by substituting the ordinary aqueous-gentian-violet stain for the unstable anilin-gentian-violet, and by using 0.25 per cent potassium carbonate solution, which to some extent clears the field, simplified and improved materially the technic of capsule staining. Finally, by using a 20 per cent copper sulphate wash instead of the potassium carbonate he found that the specimen could be dried and mounted in balsam.

Formerly capsules were found only in the exudates of infected animals, but now they are readily demonstrated in organisms growing in artificial media. Boni found that when cultures of pneumococci are smeared in egg albumin, the capsules are easily stained. Hiss secured similar results with blood serum, and by means of his more reliable methods of staining was able to determine more fully the importance of utilizing this principle in the morphological study of the pneumococcus.

By virtue of these modern procedures it is now a comparatively simple matter to demonstrate capsules on these organisms. It is no longer a question of how encapsulated pneumococci may be stained, but of how they may be most simply and reliably stained.

*Welch does not state the reasons for this belief; it is therefore difficult to refute the positive, though incomplete, observations of Guarneri. Mucin is a glycoprotein and reacts to Millon's reagent, but the solubilities differ, and capsules are more constantly noted in albuminous media than in the presence of mucin. In fact, the mucous secretions of the mouth are not a particularly favorable environment for the demonstration of capsules, whereas capsules are readily obtained in simple broth, if only the albumin or even the peptones be sufficiently increased.

The methods of Guarnieri, of Welch, and of Buerger, reliable, though in one way or another complicated, all give temporary mounts, and are thus unsatisfactory; particularly when similar or better results may be secured by simpler procedures giving permanent preparations in balsam, which may be kept for future reference, as with the copper sulphate method of Hiss.⁶

Differential staining.—These simple methods of staining encapsulated pneumococci, however, do not always suffice to differentiate the pneumococcus from other capsule-forming bacteria.* To complete this differentiation further steps are necessary. In pneumonic exudates, pneumococci, streptococci, and pneumobacilli (Friedländer) are frequently associated together. The streptococci, except possibly under especially favorable conditions, rarely show capsules. The pneumobacilli not only are encapsulated, but the short and degenerating forms are practically indistinguishable from the pneumococci. The pneumobacillus, however, decolorizes by the Gram stain; but this does not show capsules, and when these contaminated exudates are examined for the morphological determination of the presence of these species, both the capsule and the Gram stains are required, and even then the observations may not be accurately correlated. Cultural characters are more precise and final, but require time; and, furthermore, the streptococci and pneumobacilli, when present, usually predominate; they also grow so rapidly, and the pneumococci are frequently in such small numbers, that, unless exceptional precautions are taken, the pneumococci may not be found.

For these reasons efforts have been made to obtain a reliable method which would demonstrate the capsules and the Gram differential in the same preparation. This result has been noted in specimens overexposed, or exposed with heat to the anilin stain and the iodine solutions; but this is so exceptional that special methods have been devised.

For this purpose Smith¹⁵ fixes the smears of fresh sputum from pneumonia patients in the usual way by heat. The films are then

*The morphological differences in the capsules of the pneumococci, as compared with other encapsulated organisms resembling the pneumococcus, which Buerger obtained with his simple stain, and upon which he lays so much stress, depend chiefly upon the varying stages of development or degeneration and solution of the capsule, and upon the degree of decolorization. They are in no sense differential.

steamed in anilin gentian-violet and in the iodine solution. After the usual alcohol decolorization and a few seconds' exposure to a mixture of alcohol, 4 parts, ether, 6 parts, the smear is counterstained, first in aqueous eosin, then in Loeffler's methylene blue. This is followed by slight decolorization in 95 per cent alcohol, dehydration in absolute alcohol, xylol, and balsam.

Buerger² fixes the smears of encapsulated pneumococci in Müller's fluid saturated with bichloride, for one-half minute,* and washes in water. After one minute's exposure to an alcoholic solution of iodine (U. S. P.), and washing in alcohol, the smear is stained in the usual way by the Gram method—anilin-gentian-violet, Lugol's solution, alcohol decolorization—and washed in water. After counterstaining in aqueous fuchsin, the preparations are washed and examined in 2 per cent salt solution.

With these methods of Smith and of Buerger the bacterial cells, under favorable conditions, may be stained by the Gram stain and demonstrated with capsules. The procedures, however, are complicated, not as accurate and reliable as the simple capsule stains, or give temporary mounts; and none of the methods thus far considered are applicable to the demonstration of encapsulated organisms in sections of diseased tissues.

THE NEW METHODS.

In the attempt to secure methods by which the differential staining of encapsulated organisms in tissues as well as films could be easily accomplished with a reasonable degree of certainty, a great variety of procedures were tried without success before satisfactory results were finally obtained. Suffice it to note that all the efforts to keep the capsules from dissolving in the course of hardening, imbedding, and staining, by the addition of various chemicals to the solutions, so successfully adopted in the simple methods of Guarnieri and of Hiss for films, failed to give reliable results. Exceptionally, a few encapsulated organisms were stained in sections treated in this fashion, but the result of these procedures proved to be wholly beyond control, and was thus of no practical value. It was therefore evident, if the Gram stain was to be used, that the solution of the problem depended primarily upon securing a per-

* In my experience with this method longer exposures are advisable: in fact, essential.

manent fixation or coagulation of the capsule. Obviously, success in securing permanent fixation depended upon the composition of the capsule. In the absence of data to the contrary, the results of Guarnieri's chemical studies, and the fact that capsule preservation or formation is largely determined by the presence of albumin, suggested with reasonable probability an albuminous composition. Attention was therefore directed to the study of the action of bichloride of mercury and formalin, which not only precipitate albumins, but enter into chemical combination with them.

Bichloride methods.—Bichloride of mercury was tested in both aqueous and alcoholic solutions. Alcoholic solutions proved more efficient. Dried smears, fixed in saturated bichloride-alcohol for a few minutes and washed in water, may be stained in aqueous-gentian-violet, and mounted in the usual way, or they may be decolorized by retreatment in the bichloride-alcohol for a few seconds before washing in water and drying. In these preparations the encapsulated cells are often very sharply demonstrated in a perfectly clear field. With the Gram differential procedures the capsules fixed in bichloride-alcohol rarely* withstand the osmosis required to bring the specimen to a balsam mount, and as applied to the study of tissues in section it was a complete failure.

Bichloride-alcohol thus proved valuable in coagulating and partially fixing the capsules, and also in clearing the field; but, aside from this, it offered little practical advantage over the simple staining procedures of other observers.

Formalin methods.—The fixation obtained with strong solutions of formalin proved more stable. It was also found that, when desirable, formalin may be effectively used as a wash after simple staining with aqueous-gentian-violet, or 5 to 10 per cent may be added to the stain to shorten the procedure. Although useful and simple, these methods offer no special advantage over the definite fixation secured by the stronger (40 per cent) solutions of formalin,†

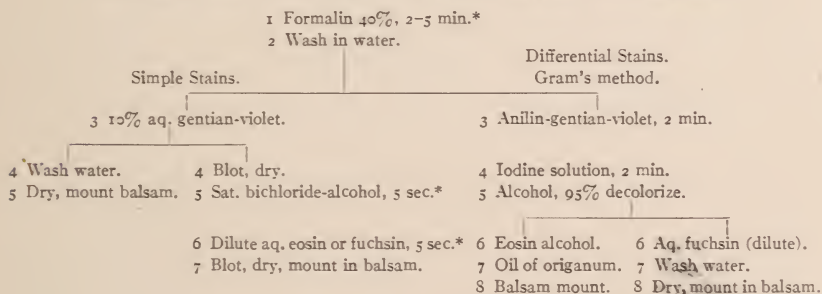
*Excellent balsam preparations for demonstration purposes were readily secured, but for routine work the results were uncertain. With other species of encapsulated bacteria this may prove more reliable for in sections of tissues hardened in bichloride Wright and Mallory¹⁷ demonstrated capsules on a Gram negative organism resembling the Friedländer bacillus.

†The addition of alkali to the formalin interfered seriously with the fixation, but the addition of acetic acid;‡ to 2 per cent in some instances proved advantageous, especially in securing penetration of the tissues, and also it was thought, by counteracting the alkaline reaction of the body tissues.

after which a great variety of staining as procedures, differential well as simple, may be readily adopted according to the character of the material to be examined, and the purposes of the examination. The simple routine gentian-violet stain may be used, or this, after drying, may be decolorized for a few seconds in saturated bichloride-alcohol and counterstained, or the Gram differential method may be employed. Finally bits of diseased tissue may be hardened in 40 per cent formalin, imbedded in celloidin, and sections cut and stained by simple and differential methods to demonstrate the presence of encapsulated organisms.

Briefly tabulated, the technic for smear preparations is as follows:

TECHNIC FOR THE FIXING AND STAINING OF SMEARS.



THE TECHNIC FOR SMEARS OF SPUTUM.

The demonstration of encapsulated pneumococci in smears of exudates by these formalin methods is thus comparatively simple. The demonstration of encapsulated pneumococci in smears of sputum, however, has proved in my experience another problem. With fresh sputum containing exudate coughed up from the lung good results may often be secured, especially if the more purulent portions of the sputum are selected for examination. But more frequently the organisms fail to show good capsule development, and in the secretions from healthy persons the capsule is even less marked. Apparently saliva and mucus offer poor conditions for capsule formation, and, although the cells coming from the lung exudates may retain their capsules for some time, the organisms growing in the mouth secretions may lose their capsules. To demon-

*Thick smears require longer exposure than thin smears of material containing few cells and little detritus.

strate capsules on such cells, albumin* in the form of blood serum (Hiss) or egg albumin (Boni) must be added, and thoroughly mixed with the sputum before the smears are made. The mucus in which many of the organisms are imbedded protects them from the albumin, and they fail to show capsules. Often it is only the isolated or exposed cells which show capsules. In the fixation by formalin the mucus also protects the cells from the action of this reagent; longer exposure is therefore required, and after this the preparation should be thoroughly washed in water, or some of the formalin will be retained and precipitate the anilin stain when this is used.

The demonstration of capsules on the pneumococci in sputum thus depends primarily upon bringing the bacterial cells into an albuminous environment favorable to the preservation or development of capsules.† This accomplished, the staining procedure is purely a matter of choice. With the formalin preparations the Gram stain may be used and a differentiation for practical purposes at once secured, as on further study in culture, Gram-positive, encapsulated organisms of pneumococcus morphology practically always give the biological characters of the pneumococcus. Occasionally encapsulated forms of the *Micrococcus tetragenus* resemble atypical pneumococci, but usually there is little difficulty in differentiating these forms. The *Streptococcus mucosus*, in my experience, cannot be differentiated morphologically from the pneumococcus. Streptococci, in my experience, have rarely given definite capsules‡ with these formalin methods.

THE TECHNIC FOR SECTIONS OF TISSUES.

Although the fixation of the pneumococcus capsule in smears is simple, in tissues hardened for celloidin sections it is difficult and less certain. The chief difficulty lies in securing sufficient penetration. In the body fluids the formalin is diluted, and it combines with the albuminous material which is coagulated. This

*Blood serum seems to mix with sputum better than egg albumin, but as suggested by Professor F. C. Wood, egg albumin, if it is diluted and made isotonic with NaCl solution, will give practically as good results.

†With some pneumococci capsules are obtained with great difficulty. For complete discussion of the differentiation of the pneumococci the reader is referred to the work of Hiss, Borden, and Knapps.

‡Occasionally a faint, hazy periphery, suggesting a shrunken or degenerated, partially dissolved capsule, was noted.

protects many of the bacterial cells from the action of the formalin, and the capsules of these cells are not properly fixed, and are thus dissolved by subsequent treatment. These difficulties are often encountered in precise histological work, and are largely eliminated by injecting the tissue with the hardening fluid, or, when this is not feasible, by cutting the material into small bits or thin slices.

The lungs are easily injected through the trachea and are quickly hardened in the distended condition. The lesions may then be cut into small pieces and the fixation completed in fresh, strong formalin, the whole process taking from three to five hours. After alcohol dehydration the material is imbedded in celloidin and cut in the usual way. It is important to have thin sections* for microscopical examination; otherwise the encapsulated bacteria lying in the exudate among the cells cannot be easily distinguished. After alcohol dehydration these sections may be fixed on the slide by partially dissolving the celloidin with ether, or alcohol and ether, equal parts. A few seconds in alcohol will then harden the thin film of celloidin covering the section, and after washing in water the preparation is ready to be stained. A variety of staining procedures may be employed;† but the Gram method, by virtue of its differentiation, has proved the most useful. Anilin-gentian-violet stain for two to five minutes, iodine solution one to two minutes, alcohol decolorization, eosin-alcohol counterstain, eosin-oil of origanum to clear, and balsam mounting, are the several steps of the technic. This has rarely failed to give good results, but occasionally the pneumococci, after prolonged exposure, or after exposure to weak or impure formalin solutions, decolorize partially in the alcohol. This technical error, when it occurred, was rectified by using a 5 per cent bichloride-alcohol for the decolorization following the iodine solution, so that the material could be studied, though not so accurately as when the cells were properly fixed. By

*Thin sections are readily secured by painting the surface of the block with dilute celloidin-ether between each stroke of the knife.

†A combination of the Nicolle and Van Gieson methods of staining has given some excellent preparations. After staining in Loeffler alkaline methylene blue, the dye is fixed in the bacterial cells by 10 per cent aqueous tannic acid. This is followed by a partial decolorization in alcohol, counterstaining in van Gieson's strong fuchsin-picric acid solution (Freeborn, *Proc. N. Y. Path. Soc.*, 1893, p. 73), differentiation in picric acid alcohol, clearing in picric acid-oil of origanum, and mounting in balsam. The Gram stain may be substituted for the methylene blue and tannic acid stain, but it is apt to decolorize in the acid alcohol. The picric acid cellular stain is more easily studied than the eosin stain.

using strong bichloride-alcohol for decolorization, Gram-negative organisms retained the gentian-violet stain and were demonstrable in the sections.

The difficulties of staining encapsulated pneumococci in sections of diseased tissues are thus purely technical, similar to those met with in all precise histological work, and with due care easily eliminated. By using the Gram method of staining a practical differential procedure is available for the accurate determination of pneumococci in sections of diseased tissues. This is particularly valuable in the study of pneumonic lesions, where contaminations or secondary infections often occur, and cultural examination fails to reveal the true significance of the bacteria isolated, or the relationship of these organisms to the disease processes.

These methods of studying the encapsulated pneumococcus, uniform in principle, for the most part simple, and adaptable to varying conditions, have been of such value in my studies that I believe they may prove similarly useful to other workers in this field.

BIBLIOGRAPHY.

1. BONI. *Munch. med. Wchnschr.*, 1900, 47, p. 1262.
2. BUERGER. *Med. News*, 1904, 85, p. 1117; *Centralbl. j. Bakt.*, 1905, orig. 39, pp. 216, 335.
3. FRIEDLÄNDER. *Fortschr. der Med.*, 1885, 2, p. 757.
4. GORDON. *Brit. Med. Jour.*, March 19, 1904, p. 659.
5. GUARNIERI. *Atti. d. v. Accad. med. di Roma.*, 1888, Ser. II, 4, p. 114.
6. HISS. *Jour. Exp. Med.*, 1904, 6, p. 335.
7. HISS, BORDEN, AND KNAPP. *Ibid.*, 1905, 7, p. 547.
8. KOLLE U. WASSERMANN. *Handbuch der pathogenen Mikroorg.*, 1903, Bd. 1, p. 422.
9. MACCONKEY. *Lancet*, 1898, 2, p. 1262.
10. MALLORY. *Ztschr. j. Hyg.*, 1895, 20, p. 220.
11. MUIR AND RITCHIE. *Manual of Bacteriology*, 1903, p. 106.
12. PANE. *Centralbl. j. Bakt.*, 1898, 24, p. 289.
13. RIBBERT. *Deutsche med. Wchnschr.*, 1885, 11, p. 136.
14. ROUX, NICOLLE ET REMLINGER. *Traité de technique microbiolog.*, 1902, p. 328.
15. SMITH. *Boston Med. and Surg. Jour.*, 1902, 147, pp. 659-67.
16. WELCH. *Johns Hopkins Hospital Bull.*, 1892, 13, p. 128.
17. WRIGHT. *Ztschr. j. Hyg.*, 1895, 20, p. 220.

LIVER NECROSIS AND VENOUS THROMBOSIS IN HORSES ACTIVELY IMMUNIZED WITH DIPHTHERIA AND TETANUS TOXINS AND WITH STREPTO- COCCI AND THEIR PRODUCTS.*†

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OUR search of the literature dealing with the anatomical and histological findings in man or animals dead of diphtheria, tetanus, or streptococcus infections, either natural or artificial, has failed to reveal to us definite records of the conditions similar to those which we are about to describe.‡

Law in his extensive work on *veterinary medicine* gives no descriptions of liver necrosis in the horse exactly comparable to the condition in our series. However, from the statements in his chapter on "Hepatic Hemorrhage and Rupture" we gather the impression that various degenerations of liver cells and subsequent traumatic hemorrhage and rupture of the capsule are far more common in the horse than in man. From his pathological and clinical descriptions the usual cases of hemorrhage and rupture are undoubtedly much milder than those in our horses, as the latter rarely live over 10 minutes after the onset of symptoms, while he gives five hours to five days as the period of duration.

Klittine¹ calls attention to fatty degeneration of the liver cells of guinea-pigs, the subjects of chronic experimental diphtheria. Dzierzowsky² also mentions the occurrence of fatty degeneration of the livers of horses which have been used in diphtheria antitoxin production.

* Read at the Sixth Annual Meeting of the American Association of Pathologists and Bacteriologists held at Baltimore, May 18, 1906.

† Received for publication June 4, 1906.

‡ That liver necroses have been frequently found in horses, the subjects of diphtheria antitoxin production, is no secret to many of those in charge of laboratories where such work is carried on, but no description of this condition has been published so far as we have been able to learn.

¹ *Arch. d. Sci. Biol.* (St. Petersburg), 1900, 8, p. 2.

² *Ibid.*, 1902, 9, p. 293.

Pettit and Girard¹ found complete necrosis of the spleen in a horse used in the production of antiplague serum.

We have to report the finding at autopsy of more or less diffuse necrosis of the liver, or thrombosis of some of the branches of the portal or splenic veins or of the pulmonary artery, either alone or in combination, in 12 horses. These animals had been subjected to repeated subcutaneous injections of the artificially produced toxins of the diphtheria or the tetanus bacillus, or of the various preparations of living or dead cultures, or of suspensions of dysentery bacilli or streptococci. In this series there are seven horses treated with diphtheria toxin, two with tetanus, two with streptococcus, and one with dysentery. A horse treated with diphtheria toxin and killed because of its crippled condition is added to the series for purposes of comparison, as its organs were found to be normal. (See Table 1.)

Superficial postmortem examinations were made of the first three diphtheria horses, and incomplete autopsies, in that no special search for thrombosis was made, were performed on the second three horses in the series. (See Table 1.)

TABLE 1.
SHOWING AUTOPSY FINDINGS RELATING TO NECROSIS AND THROMBOSIS.*

HORSE	SERVICE				LIVER		THROMBOSIS OF VEINS		
	Disease	Duration Yrs. Mos.	Sudden Death	Killed	Necrosis	Rupture	Liver	Spleen	Pulmo- nary Artery
11.....	Diphtheria	1 11	+		Diffuse	+	?	?	?
17.....	"	2 1	+		"	+	?	?	?
28.....	"	2	+		"	+	?	?	?
36.....	Dysentery	11	+		"	+	?	?	?
37.....	Streptococcus	1 9	+		"	+	?	?	?
38.....	"	1 9½	+		"	+	?	?	?
21.....	Tetanus	3 3	+		"	+	+	+	—
29.....	"	3 7	+		"	+	+	—	+
59.....	Diphtheria	3 8		+†	—	—	+	—	+
57.....	"	4		+†	—	—	—	—	—
43.....	"	2 2	+		Diffuse	+	+	+	—
50.....	"	1 7	+		Localized	—	—	—	—
56.....	"	1 5		+†	—	—	—	—	—

As we have not been fortunate enough to be present when any of the horses died from the effects of hemorrhage and rupture of the

* *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 272.

* The horses reported upon in this series include all those which have died, or have been killed, after a year and a half of service under the various treatments, since the observations were started.

† Horses 59 and 57 were killed because they were in such a disturbed state that it was impossible to control them. Horse 56 was hardly able to walk on account of the nearly immovable condition of the joints of its legs, and as its serum was of no value it was killed as a matter of economy. Its tissues were normal.

liver, we are unable to give a detailed clinical picture of the symptoms preceding death in these cases. The general conditions as described by an attendant are as follows: The animals are apparently in their usual condition. They may have recently eaten a normal meal. Attention is first attracted to them by a rapid noisy breathing. The animal exhibits signs of collapse, and shortly falls to the floor. The breathing becomes very labored and the animal struggles. This stage is followed by a rapid and progressive loss of power, and death ensues. The whole course may not last over 10 minutes.

At autopsy the peritoneal cavity contains a very large amount of fluid blood or serum and clots. The liver is usually very soft and



FIG. 1.—Necrosis, Hemorrhage, and Rupture of Liver. Horse 43.

friable, and is handled with difficulty. The capsule presents one or more ruptures, and at such places the liver substance is broken up into stringy fragments, interspersed with blood clots. (See Figure 1.)

Our attention was first called to the occurrence of thrombosis in association with diffuse necrosis of the liver by the findings at the autopsy of Horse 21, which occurred April 9, 1905. As is shown in Table 1, this animal had been in active service for the production of tetanus antitoxin for a period of over three years, and was in apparent excellent physical condition when she died in the manner already described. As the protocol of this animal gives a fairly representative picture of the conditions found in the other horses in the series, it will be given in full.

On opening the abdomen a large amount of bloody fluid escapes and many light red clots. The intestines are distended. The glands of the mesentery in the ileocolic

region are enlarged and soft. The liver for the most part presents a yellowish-gray color; within the right lobe an encapsulated hemorrhagic mass. On section it is found that the entire liver is necrotic, presenting a grayish color and a consistence resembling that of thick oatmeal. In some places there is added to this the yellowish tinge of bile. The hemorrhage in the right lobe is found to be subcapsular and infiltrates the liver substance. It is essentially a hematoma beneath the capsule. No remnant of normal liver can be made out. Everywhere with slight pressure the liver substance can be removed leaving only the connective tissue framework.

The portal veins at their entrance to the liver are everywhere filled with smooth, firm, yellowish-white clots but slightly streaked with red. (See Fig. 2.) These clots project into all the smaller branches and are definitely adherent to the vessel wall. In most instances they completely occlude the vessels in which they lie. On section



FIG. 2.—Thrombosis in Portal Vein. Horse 21.

through the liver small, firm, yellow, rigid clots protrude from the surface. The hepatic artery is free as are also the aorta and vena cava.

The spleen shows slight thickening of the capsule, and on section are found here and there a few soft swollen areas of a darker red than the surrounding tissue and in the center of each a clot similar to those found in the liver. On dissecting the main branch of the splenic vein an adherent thrombus can be followed for some distance. The splenic artery is free.

There is no accumulation of fluid in either pleural or pericardial cavity. The heart is pale, but otherwise negative. The lungs are pale and neither congested nor edematous. There are no thrombi in the pulmonary, coronary, or other vessels of the thorax. The kidneys are pale and cloudy. The adrenals are swollen and soft.

Careful examination of vascular system shows no thrombi in vessels other than those of liver and spleen.

Anatomical diagnosis.—Widespread necrosis of liver. Hematoma of the liver.

* Focal hemorrhages in the spleen. Hemoperitoneum. Thrombosis of the portal and splenic veins.

Histology, liver.—Sections taken at random from different portions of the liver show practically complete necrosis without leucocytic infiltration. Here and there the nuclei in small portions of a lobule may be distinguished, as may also those of the structures in the larger portal spaces, but otherwise the destruction is complete. Much bile pigment is present in the necrotic cells.

Sections passing through the periphery of the hematoma in the right lobe show this mass to be surrounded by a definite fibrous capsule from which prolongations pass into the adjacent necrotic tissue. In this fibrous tissue is a diffuse deposition of old blood pigment.

Other sections show loose granulation tissue penetrating and replacing the blood clot. Here and there in the older portions are newly formed bile ducts and liver cells, the latter being large, pale, and multinucleated.

Sections through five thrombi differing in size show a uniform structure—a definite network of fibrin enmeshing large numbers of leucocytes. At the periphery the fibrin is compact and hyalin, forming broad bands. Small bands of similar hyalin are found also in the other portions. Only an occasional red corpuscle is seen.

Spleen.—Section through the occluded vein shows a thrombus, similar in structure to those found in the liver and intimately adherent to the vessel wall, which is greatly thinned and infiltrated with lymphoid cells. In the tissues about the vessels a diffuse hemorrhage has occurred. Elsewhere the spleen is normal.

Lymph nodes.—The sinuses are dilated and closely packed with large, pale, endothelial cells. These are but slightly phagocytic. In the lymph nodes are many plasma cells with, however, no diminution of the lymphoid cells. Throughout the lymphoid tissue is an abundant deposition of old blood pigment.

Adrenal.—Normal.

Lung.—Congestion, moderate edema in focal areas and slight thickening of walls of alveoli.

Kidney.—Cloudy swelling of epithelium of tubules, few foci of lymphoid cell infiltration.

Heart muscle.—Cloudy swelling.

Histological diagnosis.—Necrosis of liver with repair about hematoma. Mixed thrombi of portal vessels. Thrombosis of splenic veins with perivascular hemorrhage. Endothelial hyperplasia of lymph nodes with deposition of old blood pigment. Congestion and edema of lung. Cloudy swelling of heart and kidney.

ABSTRACTS FROM CLINICAL HISTORIES AND PROTOCOL.

HORSE 11.

Clinical notes.—Horse about 10 years old. Used in the diphtheria service. Had stiff leg joints at time of entering, which gradually became worse until horse was almost constantly confined to its stall.

The maximum antitoxin strength of its serum was approximately 500 units per c.c. During the last six months of its life the horse showed progressive emaciation. After a year and 11 months of treatment the horse suddenly developed the usual symptoms of hepatic rupture, and died in a few minutes.

Autopsy notes.—A superficial examination shows diffuse necrosis and hemorrhage of the liver, with rupture of the capsule and consequent hemoperitoneum.

HORSE 17.

Clinical notes.—Sound horse of uncertain age. Diphtheria service. Always remained well and active during period of treatment of two years and one month. Antitoxic strength of serum varied from 200 to 400 units per c.c.

The animal retained its usual weight until death, which occurred suddenly after the development of the usual symptoms of hepatic rupture.

Autopsy notes.—Examination incomplete. Diffuse necrosis of the liver with hemorrhage, rupture, and hemoperitoneum.

HORSE 28.

Clinical notes.—Sound horse about 12 years old. Diphtheria service. Active during entire period of treatment of two years. Maximum antitoxic strength of serum 400 units per c.c. Gradual loss of flesh during last two months of life. Had frequent abscesses at the sites of injection with accompanying high temperatures. Died during the night.

Autopsy notes.—Examination incomplete. Diffuse necrosis of the liver, with hemorrhage, rupture, and hemoperitoneum.

HORSE 36.

Clinical notes.—Sound horse about 10 years of age. Dysentery service. Progressively increasing doses of autolyzed suspensions of surface growth of agar cultures of two strains of *B. dysenteriae* were injected alternately into this horse. They produced very severe reactions, during which the temperature would often rise to 104° F., or over, and the site of the injection would become very much swollen and painful, and the animal would refuse to eat for several days.

After a total of 496 c.c. of these autolyzed suspensions of dysentery bacilli had been administered, living broth cultures of the same strains were administered. They caused much less vigorous systemic reactions.

About seven weeks before death the animal developed a severe diarrhea, which was checked with difficulty. It also began to lose weight, and finally became much emaciated. The animal was found dead early in the morning.

Autopsy notes.—Diffuse necrosis of the liver with hemorrhage, rupture, and hemoperitoneum. Other organs, including intestinal mucous membrane, normal microscopically. Cultures from the usual organs were negative. No thrombosis was noted in any of the vessels of the liver, spleen, or lungs, but attention was not directed particularly toward this point.

Histological diagnosis.—Intense diffuse granular degeneration with irregular focal areas of necrosis in the liver.

HORSE 37.

Clinical notes.—Sound active horse of about 12 years of age. Streptococcus service. Received, in gradually increased doses, a total of about three and a half liters of autolyzed salt solution suspensions of a rabbit-virulent streptococcus, grown on the surface of large agar plates, and later about half a liter of broth cultures of the same streptococcus. Small subcutaneous abscesses followed the later injections. The animal retained its weight and activity during the treatment. It was found dead one morning.

Autopsy notes.—Diffuse necrosis of the liver with hemorrhage, rupture, and hemoperitoneum.

HORSE 38.

Clinical notes.—Streptococcus service. Age uncertain, but animal appeared to be at least 20 years old. Had somewhat crippled legs. Became emaciated during the last two months of life. The treatment was the same as was given Horse 37 and was started at the same time, except that another streptococcus was used. This animal also died during the night, about two weeks after the death of Horse 37, and the autopsy findings were in general the same as those of that animal.

HORSE 21.

Clinical notes.—Sound active mare about 16 years old. Tetanus service. Always well. Remained active and retained its weight during life. She died in the manner already described. (Full protocol will be found in the text.)

HORSE 29.

Clinical notes.—Sound active horse about 10 years of age. Tetanus service. Remained well until about two months before death, when it had frequent rises of temperature and difficulty of breathing which were directly due to the toxin injections. During the last half-year had frequent acute abscesses at sites of injection. This horse died in the manner already described.

Autopsy notes, anatomical diagnosis.—Diffuse necrosis of the liver with subcapsular hemorrhages and rupture. Hemoperitoneum. Multiple thrombi of portal veins. Multiple thrombi of pulmonary arteries. Multiple infarcts of the lungs. General lymphatic hyperplasia.

Histological diagnosis.—Necrosis, congestion, and hemorrhage of the liver. Thrombosis of portal veins and pulmonary arteries. Focal edema and congestion of the lungs with slight leucocytic exudate. Acute endothelial hyperplasia of the lymph nodes. Congestion of the spleen. Acute interstitial (non-suppurative) nephritis. Tests of liver and kidneys for amyloid changes were negative.

HORSE 59.

Clinical notes.—Diphtheria service. Sound active horse about 10 years old. After eight months of treatment was taken suddenly while in the paddock with difficulty of breathing and evident distress. Respiration became very rapid and labored, and at the same time there was very marked spasmodic tenesmus, but no dung was passed. Pulse rate about 90. The horse became weaker as the above condition continued, and finally was unable to stand, and when down struggled violently, so that it became necessary to kill him about five hours after the onset of the symptoms. The maximum antitoxic strength of the serum was 300 units. The last bleeding occurred about 10 days prior to death.

Autopsy notes, anatomical diagnosis.—Thrombosis of portal veins. Focal hemorrhages of liver and spleen.

Histological diagnosis.—Granular degeneration and focal hemorrhages of liver. Leucocytosis.

HORSE 57.

Clinical notes.—Diphtheria service. Sound horse 10 years old. This animal had lived under the same conditions as the other horses in the service for 10 months before the treatment was begun. The toxin injections were pushed with considerable vigor, so that during the four months of treatment he received 14 injections, and had an average of nearly 3.9 days of temperature over 101° F. for each injection given,

which is the maximum for any of the horses in this series. The animal appeared to stand them satisfactorily, and gave a serum of 300 units per c.c. at the second bleeding. Immediately after the third withdrawal of eight liters of blood the animal suddenly fell to the floor in a state of collapse. Nothing was thought of this, as it is not an uncommon occurrence when larger amounts of blood are withdrawn. The horse did not attempt to rise later on, and all efforts to stimulate the animal with strychnin, etc., and to support him in slings, were futile. That night and the following day he struggled violently, and could not be quieted with full doses of morphine or chloral. The next night, and also part of the following day, he rested comfortably. Later he again became violent, and as the temperature rose to 106, and as it seemed impossible to control him, he was killed in the evening. His head and shoulders were badly bruised, and as the autopsy was not performed until the next morning, the tissues and organs showed a well-developed gas bacillus infection at that time.

Autopsy notes, anatomical diagnosis.—Acute endarteritis and thrombus formation in the main trunk of the pulmonary artery. Thrombosis of intrapulmonary branch of left pulmonary artery. Atelectasis of portion of left lung. Hemorrhagic infarct of left lung. Cloudy swelling of the myocardium. Fatty transformation of the liver with evidences of gas bacillus infection. No hemorrhage or other evidence of ante-mortem necrosis was apparent.

Histology.—Complete postmortem decomposition with gas blebs in the liver and lungs. Tests for amyloid changes in all organs were negative.

HORSE 43.

Clinical notes.—Diphtheria service. Sound horse about 10 years old. Remained well and active during treatment for two years and two months. During the last few months of life was subject to abscess formation at sites of injection. Its serum showed 500 units per c.c. after three months of treatment, and varied from that point to 700 units during the next year, after which time it varied from 300 to 500 units, until three months before death it dropped suddenly to below 200 units. Death occurred in the manner already described.

Autopsy notes, anatomical diagnosis.—Extensive necrosis of the liver with multiple hemorrhages. (See Fig. 1.) Thrombosis of small branch of the portal vein in the right lobe. Rupture of encapsulated hemorrhages in the peritoneum. Hemoperitoneum. Thrombosis of the splenic veins. Hemorrhage and edema of gastrohepatic lymph nodes. Chronic peritonitis.

Histological diagnosis.—Necrosis and amyloid degeneration of the liver, with moderate regeneration. Chronic hemorrhagic cysts of the liver. Organizing thrombi of portal and splenic veins. Amyloid degeneration of Malpighian bodies of spleen. Proliferation of intima of splenic arteries. Hemorrhage and endothelial hyperplasia of gastrohepatic lymph nodes.

HORSE 50.

Clinical notes.—Diphtheria service. Horse of uncertain age, of good condition on entering. Gradually became almost helpless through stiffness of joints and blindness. Had frequent abscess formations at sites of injections. Antitoxic strength of serum never over 300 units per c.c., and usually from 200 to 250 units. Was found dead one morning, and stall showed evidences of continued struggling. The front of the stall was covered with frothy mucus. The coughing-up of this material has never been observed in the horses dying from rupture of the liver.

Autopsy notes, anatomical diagnosis.—Necrosis and hemorrhage of left lobe of the liver. Congestion of lungs and the right lobe of the liver. General lymphatic hyperplasia. Abscess of subcutaneous tissues of back. Cloudy swelling of heart and kidneys. Acute endarteritis of left renal artery. Thickening of intima of splenic artery. Focal lesions of adrenals. Chronic peritonitis.

Histological diagnosis.—Congestion, edema, hemorrhage, amyloid degeneration, and necrosis of the liver. Chronic interstitial myocarditis. Subpericardial hemorrhage with hyalin degeneration of muscle fibers, and leucocytic infiltration. Edema and congestion of lungs with hemorrhages. Edema and hemorrhages of lymph nodes. Amyloid transformation of Malpighian bodies of the spleen. Localized endarteritis and mesarteritis of left renal artery.

HORSE 56.

Clinical notes.—Diphtheria service. Horse about nine years old on entering. Developed stiffness of joints and became badly crippled. Antitoxic strength of its serum was rarely up to 300 units per c.c., and usually showed but 200 units. After a year and five months of treatment the animal was killed.

Autopsy notes.—Macroscopically and microscopically the liver, spleen, kidney, pancreas, adrenals, lymph nodes, lungs, and heart muscle appeared to be normal.

Table 1 presents a summary of the findings relating to necrosis of the liver and thrombosis in 12 horses which have presented such changes, and also gives those of one horse in whom all the organs were normal, although it had been subjected to active treatment with diphtheria toxin for a period of a year and five months. The other changes found in the tissues of these 12 horses varied to some extent, but such changes did not differ greatly from those to be found in the tissues of fatal cases of diphtheria in man, such as endothelial hyperplasia of lymph nodes, and cloudy swelling of kidney epithelium and heart muscle, etc.

One or two unusual findings were, however, observed. In Horses 43 and 50, belonging to the diphtheria service, amyloid changes were observed in both liver and spleen, and especially in the latter, but were not present in the kidney, heart muscle, or lymph nodes. These animals had developed an unusual number of subcutaneous abscesses at the sites of the toxin injections during the later months of their lives.

In the liver in tetanus horse 21 around the margin of a subcapsular hematoma, and in the necrotic liver of diphtheria horse 43 about the larger portal spaces were found evidences of attempted regeneration in the way of newly formed false bile ducts, and, especially in the latter horse, of columns of newly formed liver cells. In the ne-

crotic livers of the other horses there were no evidences of repair. Also in the tetanus horse 29 there were evidences of beginning interstitial nephritis, of the acute non-suppurative type found in diphtheria of man, accompanying widespread disintegration of the epithelium of convoluted tubules.

A study of Table 1 will show that horses presenting necrosis of the liver did not, in all cases, show thrombosis in any organ, nor in animals presenting both conditions was the thrombosis in the hepatic veins always as extensive in its distribution as the necrosis. On the other hand, two diphtheria horses presented thrombosis without evidences of liver necrosis. Unless, therefore, the thrombosis in some instances is secondary and dependent upon the necrosis, and in other cases upon other conditions, we cannot attach much significance to their fairly close association.

That the necrosis is not usually of sudden or rapid development is shown by the fact that the livers of two horses, tetanus horse 21 and diphtheria horse 43, presented encapsulated hematomas of various sizes scattered throughout the liver substance. The thickness of the capsules and the character of their contents indicated their formation at least several months prior to death, which in turn demonstrated the still earlier existence of extensive necrosis.

With the object of ascertaining whether there existed any common factor in the clinical histories of these horses which might assist in arriving at either the etiology of the liver necrosis or the thrombosis, or of both, the individual factors in the history of each horse which has died since these conditions were noted, have been worked out and are compared in Tables 1, 2, 3, and 4.

It is evident from the fact that some horses in the diphtheria, tetanus, streptococcus, and dysentery services presented diffuse necrosis of the liver, that this condition is not due to any special pathogenic property of any one of the organisms used in the immunizations. If, however, it is the result of the activity of some bacterial property, it is not necessary to suppose that the agent in all cases belongs to either the soluble toxins or the so-called bacterial endotoxins, for the reason that the tetanus horses received only toxin which had been filtered through Pasteur-Chamberlain filters, and consequently no bacterial bodies were injected; and, on the other

hand, the streptococcus horses which received large amounts of dead cultures were equally affected.

It hardly seems probable that any of the constituents of the various media used in the preparation of the toxins and toxic products, such as the meat extractives, peptone, etc., could be the causative agent of the liver necrosis, for the reason that the two horses of the streptococcus service (37 and 38) received only about 500 c.c. of broth containing such materials during a period of about one and three-quarters years, the later other injections consisting of suspensions of bacteria or their bodies in physiological salt solution. (See Table 2.)

Neither can any effect produced by the repeated abstraction of blood from such horses be the main cause of the liver necrosis, for, as is seen in Table 2, Horse 36 in the dysentery service was bled only one liter on a single occasion, and the streptococcus horses 37 and 38 had bled eight liters, abstracted on only six and seven occasions respectively during a period of a year and three-quarters. On the other hand, Horse 56 of the diphtheria service was bled eight liters on 18 occasions during a period of a year and a half, and at death its liver was absolutely normal.

TABLE 2.
SHOWING RELATION OF THE MATERIALS INJECTED AND OF REPEATED BLEEDINGS TO LIVER NECROSIS.

Horse	Toxic Broth Injected	Autolyzed or Dead Bacterial Suspensions	Amount Carbolic Acid Injected	Number of Bleedings (8 Liters Each)	Condition at Death	Extent of Necrosis
11.....	42 liters		210 ± c.c.	21	Poor	Extensive
17.....	50 "		295 ±	25	Good	"
28.....	38 "		190 ±	20	Poor	"
36.....		800 c.c.	2½ ±	0	"	"
37.....		4 liters	17 ±	6	Good	"
38.....		4½ "	19 ±	7	Poor	"
21.....	63 liters		165 ±	20	Good	"
29.....	78 "		235 ±	35	"	"
43.....	77 "		385 ±	25	"	"
50.....	50 "		250 ±	15	Poor	Left lobe
56.....	33 "		165 ±	18	"	No necrosis

It is likewise possible to exclude the antiseptics used in the preservation of the bacterial toxic agents as an important factor in the production of liver necrosis, although nearly all the toxic materials administered contained from 0.3 to 0.5 per cent of either carbolic acid or trikresol. A study of Table 2 will show that the actual amounts of these antiseptics received by the different horses varied

from about 2.5 c.c. during 11 months in the case of dysentery horse 36, and about 17 and 19 c.c. in the streptococcus horses 37 and 38 respectively, to 165 and 235 c.c. in the tetanus horses 21 and 29 respectively, and from 190 to 385 c.c. in the various diphtheria horses presenting necrosis of the liver. In other words, the horses receiving the larger amounts of antiseptics lived the longer periods.

Nor could we explain the necrosis of the liver by any general disturbance of metabolism due to the injections of the bacterial toxic agents, for some of the animals which showed the most diffuse necrosis were to all outward appearances and according to their weight in excellent physical condition, as is indicated in Table 2.

There is also no apparent reason for believing that the conditions under which such horses are kept in antitoxin stables is the main factor in the causation of the necrosis. Diphtheria horse 56 was kept under the same conditions as the others for a period of a year and a half, and at autopsy the liver was normal.

A careful study of the temperature charts and a knowledge of the manner in which the horses withstood the injections will, perhaps, give as likely an explanation of the causation of the necrosis as can be found. In Table 3 will be found the number of days during the treatment of each horse, in which the temperature stood at the various points from 101° to 105° + Fahrenheit. It will be seen that a fair degree of uniformity exists in this number of days of fever in the animals which had necrosis of the liver. These increases of tem-

TABLE 3.
SHOWING RELATION OF REACTIONS DUE TO TOXINS INJECTED TO LIVER NECROSIS.

HORSE	DISEASE	DURATION OF TREATMENT	NUMBER INJECTIONS	DAYS OF TEMP. OVER 101° FAHR.					AVER. DAYS OF TEMPERATURE FOR EACH INJECTION
				101 to 102	102 to 103	103 to 104	104 to 105	Total	
		Yrs. Mos.							"
11.....	Diphtheria	1 11	75	95	73	14	1	183	2.4
17.....	"	2 1	77	85	75	22	2	184	2.4
28.....	"	2	69	92	74	38	9	213	3.1
36.....	Dysentery	11	46	86	62	14	4	166	3.6
37.....	Streptococcus	1 9	54	76	44	19	2	141	2.6
38.....	"	1 9½	60	98	48	5	1	152	2.5
21.....	Tetanus	3 3	126	95	69	9	1	174	1.4
29.....	"	3 7	138	135	53	8	2	198	1.4
43.....	Diphtheria	2 2	77	92	65	31	3	191	2.2
50.....	"	1 7	64	79	39	20	0	138	2.1
56*.....	"	1 5	59	63	57	34	2	146	2.4

* No liver necrosis in this horse.

perature cannot be accepted, however, as an exact index of the character of the reaction, as some horses commonly have considerable fever without any other manifestation of serious disturbance after toxin injections. However, the variations in the temperature curves are due largely to the type of immunization. Thus the dysentery horse had apparently fewer days of temperature than those used in the diphtheria work, but he lived under the treatment only about one-half as long as the latter. It is well known that horses are particularly susceptible to injections of cultures of true or paradyentery bacilli; and this horse withstood the injections very poorly. On the other hand, the horses treated with injections of even large doses of tetanus toxin show only slight disturbances, and in harmony with this is the long duration of the period of treatment in Horses 21 and 29, each of which lived considerably over three years. The two streptococcus horses withstood the injections about as well as the diphtheria animals. Each lived approximately a year and three-quarters, and that was nearly the average life, a year and 11 months, of the five diphtheria horses.

As the horses used in each service lived approximately the same length of time under their respective treatments as, and as the animals receiving the injections of the toxins causing the milder reactions lived for considerably longer periods than, did those receiving toxins producing the most violent reactions, and as we have excluded other possible factors, we are led to the conclusion that the liver necrosis is, without much doubt, mainly the effect of the administration of repeated doses of the bacterial products.

That some of the other factors which appear to be excluded by our findings may be contributory to the main causative agent in some instances cannot be denied.

A possible relationship between the effect of the toxic products of these bacteria on the liver cells and the production of antitoxic or other anti-bodies is not capable of either positive or negative determination from the available data.

In the diphtheria and tetanus horses under consideration no relationship can be made out between the course of the necrotic changes in the liver and the well-known progressive decrease in the antitoxic strength of the sera of some horses after prolonged treat-

ment. Thus diphtheria horse 50, which had necrosis of the liver, gave an antitoxin of from 300 to 350 units per cubic centimeter in only two early bleedings out of a total of 15, the others producing sera of from 200 to 250 units. On the other hand Horse 56, which showed a normal liver when killed after an immunization life of one year and five months, gave almost identically the same grades of serum in 17 bleedings. That is, the early bleedings gave sera of 400 units, and those subsequently from 300 down to 200 units, which was the strength at the time the animal was killed. These findings would seem to indicate that the customary decrease in antitoxic power, which occurs in the sera of many horses as their immunization proceeds after the first few months, is not exclusively due to a progressive liver necrosis. However, the amount of data obtained does not permit us to draw any conclusions concerning the possible relation of the liver to antitoxin production. The bleedings are too infrequent, and the continuation of the antitoxic power of the serum is probably dependent upon so many factors that the absence of liver necrosis in one horse whose serum had shown the usual progressive loss of power, is entirely insufficient evidence to warrant the exclusion of the liver as a factor in antitoxin production.

Brunton and Bokenham¹ have endeavored to show that the liver is capable of producing diphtheria antitoxin by the action of toxin passed through the portal venous system. While it might be questioned whether they have clearly proven the formation of true antitoxin, they undoubtedly showed that the liver cells have an affinity for pure diphtheria toxin, and are capable of at least greatly reducing the poisonous power of the crude product.

A similar affinity of the liver cells of some invertebrates for tetanus toxin has been claimed by Metchnikoff.² He states that in scorpions injected with tetanus toxin the liver is the only organ which absorbs and retains the toxin.

If the liver is capable of absorbing large amounts of the toxic bacterial products, and if their direct action on it is the means of producing liver necrosis, then in most horses treated with such agents the liver is very probably capable of disposing of large amounts of these products without injury; for the liver necrosis is not present

¹ *Jour. Path. and Bact.*, 1904, 10, p. 50.

² *L'Immunité*, etc., Paris, 1901, p. 343.

in many horses, at least, which have come to autopsy after injections of considerable amounts of diphtheria toxin distributed over several months. (See history of Horse 56.)

In view of recent work on autolysis of liver tissues *in vitro*, it may not be out of place to consider the possibility that this late necrosis is due to alterations or disturbances of those factors which prevent the action of the autolytic ferments normally present in the liver.

Baer and Loeb¹ have shown that weak alkalis retard and weak acids stimulate the autolytic ferments in the liver, and that the elements in normal serum which strongly retard or prevent autolysis are the serum albumen and the fibrinogen, while the serum globulin has the opposite effect, namely, a strong stimulating action on the autolytic ferment.

Hiss and Atkinson,² Butjagin,³ and others claim that an increase in the globulin content of the blood occurs during active immunization, although this is disputed by Müller.⁴

Butjagin also demonstrated a gradual decline in the alkalinity of the blood during the later part of the period of immunization.

In view of the work of Baer and Loeb, the occurrence of these or other similar blood changes may have some relation to the development of the liver necrosis or autolysis.

THROMBOSIS.

Of the horses upon which careful autopsies were performed and which presented necroses of the liver, extensive coexisting thrombosis of the veins of that organ was found in one (Horse 21), and to a lesser extent in two others (29 and 43). In the horses treated with streptococcal and dysenteric bacterial products, no careful searches of the entire substance of their livers with this point in view were made.

In one diphtheria (43) and in one tetanus (21) horse the veins of the spleen were likewise the seat of thrombosis to a greater or less extent. The other tetanus horse (29) had extensive thrombosis of the pulmonary arteries and infarction of the lungs.

As has been stated, two diphtheria horses had thrombosis occur-

¹ *Arch. f. exper. Path. u. Phar.*, 1905, 53, p. 1.

² *Jour. Exper. Med.*, 1900, 5, p. 47.

³ *Hyg. Rund.*, 1902, 12, p. 1193.

⁴ *Beiträge z. chem. Physiol. u. Path.*, 1905, 6, p. 454.

ring without necrotic changes in the liver. In one (59) the thrombosis was confined to three large branches of the portal vein in the liver, while in the other (57) the thrombosis was present in the main branch of the pulmonary artery and in the intrapulmonary branch of the left pulmonary artery. In the latter horse the clinical manifestations arose immediately after a withdrawal of the usual eight liters of blood, and may have been due to the formation of a blood clot at the site of this operation and the detachment and passage of the clot into the venous circulation. The thrombus in the main pulmonary artery was in the form of a pedunculated vegetation about the size of a walnut. The horse apparently suffered considerable thoracic pain, and was so weak as to be unable to stand even with the assistance of slings.

The other horse (59) with localized thrombosis of the portal veins gave indications of great pain in the abdominal cavity, with tenesmus, and also complete prostration and excessively rapid respiration and a high pulse rate. After the continuation of these symptoms for about five hours the animal was killed, and the autopsy performed immediately.

If the conditions operating to produce the thrombosis in this case are the same as in those where the thrombosis coexisted with liver necrosis, it is remarkable that it should have occurred as early as the eighth month of the immunization period, and that the clinical symptoms should be so much more severe than in those cases where thrombosis occurred later and more extensively.

In looking for some factor or condition operative during the period of treatment which might be considered as of etiological significance for the thrombosis, we are not afforded as much data as we would like, owing to the failure to examine and note particularly the presence or absence of thrombi in the first few animals which died from rupture of the liver with hemorrhage. Thus we have no definite information on this point in three diphtheria, one dysentery, and two streptococcus horses.

From the data tabulated in Table 4 it can be seen that by excluding the horses with early thrombosis the treatment given and its effects on the three remaining horses are quite similar. The only exception is to be found in a considerable difference in the amounts

TABLE 4.
RELATION OF THE TREATMENT OF HORSES TO THE DEVELOPMENT OF THROMBOSIS.

No.	Diseases	Immunization Period	Liters of Broth Injected	Amount of Carbolic Acid Injected	Days of Tem. over 101°	Number of Bleedings of 8 Liters Each
50.....	Diphtheria	8 mos.	16½	82 ± c.c.	91	7
57.....	"	4 "	5½	27 ±	54	43
21.....	Tetanus	3 yrs. 3 "	63	165 ±	174	29
29.....	"	3 " 7 "	78	235 ±	198	35
43.....	Diphtheria	2 " 2 "	77	385 ±	191	25

of carbolic acid or trikresol which the horses received with the toxins injected. We are of the opinion that these antiseptics played no essential part in the production of the conditions favoring thrombosis.

We cannot, however, readily exclude in this way a possible influence favoring thrombosis by the injection of those elements which make up the broth used for toxin production, such as peptone meat extractions, etc. Likewise we cannot exclude the effects of the repeated abstraction of eight liters of blood every three or four weeks. We are unable, therefore, by any process of exclusion to form any definite opinion of the part played in the thrombosis by the pathogenic activity of the bacterial toxins injected into these horses.

If, however, we ascribe to the early thromboses the same etiological conditions as bring about the late thromboses, then we can find no common factor in the treatment of our horses which might have been operative in the determination of this condition.

There are, however, some noteworthy features in the relation of thrombosis to the liver necrosis in some of the horses.

Thus in Horse 50, which was the only animal with necrosis of the liver which was thoroughly examined for thrombi and none found, the necrosis and hemorrhage of the liver was not general but localized almost exclusively in the left lobe, and was evidently not so far advanced as in the other animals. As the autopsy also revealed myocarditis with small subpericardial hemorrhages, and amyloid degeneration of the liver and spleen, local endarteritis, edema, and congestion of the lungs with small hemorrhages, it is not unlikely that the animal did not die from the effects of the liver necrosis, especially as there was no rupture of that organ. It is not improbable, therefore, that thrombosis would also have occurred in this horse if he had lived longer.

In Horse 29 the portal veins in which thrombi were found corresponded closely with the areas of necrosis in the liver, and it was noted that the thrombosis seemed to proceed from the smaller veins outward into the larger branches, ending there in large, round-ended clots.

In Horse 43 the extensively necrotic liver showed thrombosis only in one small portion of the right lobe, but considerable thrombosis occurred in the spleen.

Thus, taking the findings as a whole, it can be stated that while all necrotic areas in the liver did not have coexisting thrombosis, there were no thrombi in the portal veins other than in the necrotic portions, except in the two horses already described which died with early thrombosis without necrosis. It is possible, therefore, that in some animals, at least, the portal thrombosis may be caused by lesions of the vessel walls secondary to the liver necrosis.

It is the opinion of Smith¹ that the pathological effects of the periodic losses of blood in antitoxin horses lead in certain cases to serious derangements and death. He shows that in many horses which have been bled five liters every three or four weeks for periods of from nine months to over four years, the red blood corpuscles have a reduced osmotic tension. He believes that this condition is due to the repeated bleedings and not to the toxin injections.

In Pettit and Girard's case of necrosis of the spleen in an anti-plague horse, the authors express the qualified opinion that the necrosis was not due to the periodic bleedings.

In so far as necrosis of the liver is concerned, our data do not warrant the conclusion that the periodic bleedings have any special etiological relation to it. If, therefore, the lowered osmotic tension is due to the repeated losses of blood, it is not probable that such lowered tension is in any way responsible for the liver necrosis. Whether it is or is not a factor in the causation of the thrombosis we are not prepared to state.

Butjagin has shown the presence of this phenomenon and also the existence of such alterations in the blood serum as an increase in the specific gravity, changes in albumen and globulin content, electrical conductivity, alkalinity, and refractive index during the immunizing process.

¹ *Jour. Med. Res.*, 1904, 12, p. 385

Similar work on one or more of these aspects of the subject has been reported by Müller¹ and others.

What relation exists between these alterations in the morphological, chemical, and physical conditions of the blood constituents and the pathological conditions noted by us, remains to be solved.

SUMMARY.

In a series of 12 horses, actively immunized with the products of certain pathogenic microorganisms, autopsies revealed widespread liver necrosis in nine, and localized liver necrosis in one.

Thrombosis of the portal veins was present in three, of the splenic vein in two, and of branches of the pulmonary artery in one, of the nine horses showing widespread necrosis. One horse had thrombosis of branches of the portal veins and another of the pulmonary artery without liver necrosis. Six horses were not carefully examined for thrombosis.

The evidence obtained tends to exclude the materials such as peptone, beef extractives, and antiseptics, such as carbolic acid, injected with the toxins, and also the repeated bleedings practiced, from any important rôle in the production of the liver necrosis. On the other hand, the evidence leads to the conclusion that the toxic bacterial products injected are directly or indirectly responsible for the production of this pathological condition.

The evidence is not sufficient to determine which, if any, of these factors is responsible for the occurrence of the venous thrombosis. A possible etiological action by the antiseptics injected is excluded. In some cases the portal thrombosis may result from the liver necrosis.

NOTE.—Since this paper went to press another horse (No. 60) has died after a year and four months of treatment. During the first four months of this period it received diphtheria toxin, and after that until death small doses of tetanus toxin. The horse had a very slightly varying temperature of from 104° to 106° during the two weeks prior to death, and gradually became weaker during the last few days of life, and died during the night. Just prior to the onset of the high temperature an edematous condition of the genital sheath developed, followed by a tremendous increase in the size of the penis, apparently due to venous stasis. Later the use of the left hind leg became impaired.

At autopsy thrombosis of the left iliac vein, the inferior vena cava, the veins of the tissues about the penis, the splenic vein outside the spleen, the portal vein, and some of its branches in the liver, and of some of the pulmonary arteries was found. There was no evidence of necrosis in the liver. There was a general hyperplasia of the lymph nodes.

¹ *Loc. cit.*

A FUNDAMENTAL ERROR IN CURRENT ATTEMPTS TO APPLY PHYSICAL CHEMISTRY TO SERUM PATHOLOGY.*

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IN so far as attempts to isolate bacterial toxins, antitoxins, and other specific immunity substances have proved unsuccessful, any indirect method by which light may be thrown on the nature of these important substances is welcomed by the biological chemist. The method that seems to promise most is the method of physical chemistry. The application of physico-chemical laws, therefore, has deservedly received the attention of numerous serum pathologists during the last few years.

The law of physical chemistry, whose application has apparently met with the greatest success, is the law of mass-action. When one examines, however, the experimental data on which the application of this law is based, one is struck with the numerous hypotheses such attempts necessitate. One hypothesis especially is fundamental in all these attempts. This hypothesis not only rests on no experimental basis, but experimental verification of it is practically out of the question, till toxins, antitoxins, and the like are isolated in a state of purity. It is the purpose of the present paper to point out this fundamental error in pathological reasoning, and to emphasize the vicious rôle it is playing in current biological investigation.

The law of mass-action is well established for simple chemical substances. It has been shown, for instance, that if a certain amount of substance, dissolved in a given volume of solvent, undergoes chemical change at a given rate, twice the amount of the same substance, dissolved in the same volume of solvent, will change twice as rapidly; three times the amount, three times as rapidly; and so on; the amount changing per unit time being proportional to the amount present in the solution.

*Work aided by the Rockefeller Institute for Medical Research. Received for publication April 5, 1906.

To illustrate, if in a solution containing 100 grams of cane sugar, 6 grams are hydrolyzed per minute to form glucose and fructose, then, other conditions being identical, in a solution of like volume containing but 50 grams, only half of 6 grams, or 3 grams, will be hydrolyzed per minute; and in a solution containing 200 grams, twice 6 grams, or 12 grams per minute.

This does not mean that in the latter case 12 grams will be decomposed during every minute, but only that 12 grams will decompose during the first minute. At the beginning of the second minute, there will be present in the solution but 188 grams of unchanged sugar. During that minute, therefore, there will be decomposed but $\frac{188}{200}$ of 12 grams, or 11.28 grams. During the third minute, $\frac{176.72}{200}$ of 12 grams, or 10.6 grams. And so on.

The law of mass-action, as applied to the decomposition of cane sugar, may be expressed mathematically as follows:

$$[\text{Glucose}] = K \times [\text{Cane sugar}],$$

where the symbol [Glucose] stands for the amount of glucose formed per minute, the symbol [Cane sugar] for the amount of unchanged cane sugar present at the beginning of the minute, and K a constant whose value is determined by such things as the amount and nature of the solvent, the temperature, acidity, alkalinity, and the like. In the above illustration, $K = 6$ per cent.

This formula illustrates the law of mass-action as applied to a chemical change involving but a single chemical substance. If two chemical substances enter into combination, the law is slightly more complex. Here, the rate of reaction is proportional to the amount of each substance present in the solution, and is therefore proportional to their product.

Thus, if 100 grams of methyl acetate and 10 grams of potassium hydrate are dissolved in a liter of water, they will slowly react to form potassium acetate and methyl alcohol. If, under certain conditions, three grams of potassium acetate are formed during the first unit of time, doubling the amount of either the methyl acetate or the potassium hydrate present, will double the amount of the potassium acetate so formed, and doubling the amount of both will increase

the amount fourfold. This law may be expressed mathematically as follows:

$$[\text{Potassium acetate}] = K \times [\text{Methyl acetate}] \times [\text{Potassium hydrate}],$$

where the symbol [Potassium acetate] stands for the amount of potassium acetate formed per minute, the symbols [Methyl acetate] and [Potassium hydrate] for the amounts of the reacting substances present in the solution at the beginning of that minute, and K a constant, whose value is determined by such things as the volume and nature of the solvent, the temperature, and the like. In the above illustration, $K = 0.3$ per cent.

These simple quantitative laws are readily applicable to the determination of molecular conditions in simple solutions. Most attempts to apply physical chemistry to pathology have been attempts to apply them, in a similar way, to complex biological solutions.

Probably the simplest illustration of such application is afforded by a study of ionisation. Thus, if NaCl is dissolved in water, its molecules, according to the newer chemistry, at once begin to break up, forming free atoms of Na and Cl . The rate of formation of these ions is determined by a law similar to that for the decomposition of cane sugar, and is expressed mathematically as follows:

$$[\text{Na}] = [\text{Cl}] = K \times [\text{NaCl}],$$

where $[\text{Na}]$ and $[\text{Cl}]$ represent the number of free Na - and Cl -ions formed per unit of time, $[\text{NaCl}]$ the number of unchanged NaCl molecules present in the solution at the beginning of that unit, and K a constant.

The ions so formed at once begin to recombine, forming anew molecular NaCl . The rate of recombination is determined by a law similar to that governing the reaction of methyl acetate and potassium hydrate, and is expressed mathematically as follows:

$$[\text{NaCl}] = K' \times [\text{Na}] \times [\text{Cl}],$$

where $[\text{NaCl}]$ represents the number of molecules of NaCl re-formed in unit time, $[\text{Na}]$ and $[\text{Cl}]$ the number of uncombined Na - and Cl -ions present in the solution at the beginning of that unit, and K' a constant.

At first the amount of NaCl that decomposes per unit time is in excess of the amount re-formed in the same time; but, as larger

and larger numbers of Na- and Cl-ions come to exist in the solution, the two rates become more nearly equal. Eventually a stage is reached in which new molecules are being formed as fast as old ones are being decomposed, that is, in which

$$K' \times [\text{Na}] \times [\text{Cl}] = K \times [\text{NaCl}] .$$

This may be transposed to read

$$\frac{[\text{Na}] \times [\text{Cl}]}{[\text{NaCl}]} = \frac{K}{K'} = K .$$

This equation expresses the numerical relation between the number of free and combined Na- and Cl-atoms in a simple NaCl-solution. It is one of the simplest formulæ whose application has been attempted in serum pathology. It has been conceived, for example, that the hemolytic substance ("hemolysin") present in hemolytic serum is a compound substance, formed by a union between the thermostable substance (amboceptor) and the thermolabile substance (complement) of such serum. It has been conceived that the union between these two components is similar to that between the Na- and Cl-atoms above, a state of equilibrium existing in which

$$\frac{[\text{Free amboceptor}] \times [\text{Free complement}]}{[\text{Combined amboceptor-complement}]} = K .$$

As an example of a more complex formula governing molecular distribution there may be cited the law governing the interaction of acids and bases. Here the acids and bases tend to form salts; but the salts so formed in turn tend to decompose, re-forming directly or indirectly the original acids and bases. When strong acids and strong bases are used, the tendency to decompose is practically negligible, when compared with the vigorous tendency to unite. With weak acids and weak bases, however, the tendency to decompose plays a relatively important rôle. Here it has been shown that, when certain weak acids and weak bases are put together, equilibrium is established in accordance with the formula

$$\frac{[\text{Free acid}] \times [\text{Free base}]}{[\text{Combined acid-base}]^2} = K .$$

This formula also has been applied in serum pathology. It has been conceived, for instance, that the interaction between toxins and antitoxins is similar to that between weak acids and weak bases,

and that in mixtures of the two, equilibrium is established in accordance with the formula

$$\frac{[\text{Free Toxin}] \times [\text{Free antitoxin}]}{[\text{Combined toxin-antitoxin}]^2} = K.$$

Before attempting to examine the experimental evidence cited in support of these and similar formulæ, it will be necessary to point out certain fundamental properties of simple solutions to which the formulæ are demonstrably applicable. In order to get convenient numbers for calculation, let us suppose that 702 grams of NaCl (= 12 gram-molecules) are dissolved in a liter of water. How much of the NaCl will exist in the molecular form? How much as ionic Na and Cl?

As developed above, equilibrium will be established in accordance with the formula

$$\frac{[\text{Na}] \times [\text{Cl}]}{[\text{NaCl}]} = K.$$

It is found experimentally that for NaCl, at ordinary temperatures, $K = 1$, provided $[\text{Na}]$ and $[\text{Cl}]$ are expressed as the number of gram-ions per liter, and $[\text{NaCl}]$ as the number of gram-molecules per liter. If therefore x gram-molecules of NaCl are conceived to be ionised, there will be formed x gram-ions of Na and x gram-ions of Cl, and will be left in the solution $(12-x)$ gram-molecules of un-ionised NaCl. Substituting these values in the above formula, we obtain

$$\frac{x \times x}{12 - x} = 1,$$

from which

$$x^2 + x = 12, \quad \text{or } x = 3.$$

When equilibrium is established, we therefore have three gram-molecules of NaCl ionised and $12 - 3$, or nine, gram-molecules in the molecular form. This gives rise to the verification formula

$$\frac{3 \times 3}{9} = 1.$$

Let us now suppose that two gram-ions of Cl are removed from the solution, as can readily be done by precipitation methods. What change will this bring about in the ionisation?

It is evident that equilibrium will no longer exist, since

$$\frac{3 \times (3-2)}{9} = \frac{3 \times 1}{9} \text{ is not } = 1.$$

To restore equilibrium further ionisation must take place, so as to increase the factors in the numerator and decrease the denominator. If y gram-molecules of NaCl are broken up in this secondary ionisation, there would then exist in the solution $(3+y)$ gram-ions of Na, $(1+y)$ gram-ions of Cl, and there would be left $(9-y)$ gram molecules of NaCl. The equilibrium formula would then be

$$\frac{(3+y) \times (1+y)}{(9-y)} = 1,$$

from which

$$y^2 + 5y = 6, \text{ or } y = 1.$$

This gives rise to the verification formula

$$\frac{(3+1) \times (1+1)}{9-1} = \frac{4 \times 2}{8} = 1.$$

To restore equilibrium, therefore, one additional gram-molecule of NaCl has to be ionised.

If now more ionic Cl is removed from the solution, further ionisation will necessarily take place. This will give rise to such formulæ as

$$\frac{6 \times 1}{6} = 1, \quad \frac{8 \times \frac{1}{2}}{4} = 1, \quad \frac{9 \times \frac{1}{3}}{3} = 1, \quad \frac{10 \times \frac{1}{5}}{2} = 1, \quad \frac{11 \times \frac{1}{11}}{1} = 1, \text{ etc.}$$

In this way it is possible to remove practically all the free Cl from the solution and have equilibrium constantly preserved by changes in ionic distribution.

A second fundamental fact, an understanding of which is necessary for our present purpose, is the fact that widely different solutions may contain the same amount of a certain molecular or ionic component. The following formulæ represent solutions of NaCl, each containing four gram-ions of Cl, but in no two of which the other components are equal:

$$\frac{1 \times 4}{1} = 1, \quad \frac{1 \times 4}{4} = 1, \quad \frac{4 \times 4}{16} = 1, \quad \frac{10 \times 4}{40} = 1.$$

If from each of these solutions the four gram-ions of Cl are removed, readjustment will take place as above. In so far, however,

as the amount of molecular NaCl differs in the four solutions, the amount of ionic Cl available for this readjustment, is not the same in the four solutions. From the first solution practically five gram-ions of Cl could be obtained by successive withdrawals; from the second, eight gram-ions; from the third, 20; and so on.

Although the amount of free Cl is the same in the four solutions, the amount of ionic Cl available for chemical action, differs widely. This includes not only the free Cl already present, but the potential Cl that may be formed from further dissociation of the molecular NaCl.

In a similar way solutions may contain the same amount of molecular NaCl, but widely different amounts of other elements. This is illustrated by the formulæ

$$\frac{\frac{1}{2} \times 24}{12} = 1, \quad \frac{1 \times 12}{12} = 1, \quad \frac{2 \times 6}{12} = 1, \quad \frac{3 \times 4}{12} = 1, \quad \text{etc.}$$

If part or all of the molecular NaCl were removed from these solutions, equilibrium would be restored by a recombination of free Na- and Cl-ions. In this way, by successive withdrawals of NaCl-molecules, there could be obtained from the first solution practically $12\frac{1}{2}$ gram-molecules of NaCl; from the second, 13 gram-molecules; from the third, 14; and so on.

The amount of molecular NaCl available for chemical purposes would include, not only the molecular NaCl already present, but the potential NaCl that could be formed from the recombination of the free Na- and Cl-ions present.

The error current in practically all attempts to apply formulæ of mass-action to serum pathology is the error of neglecting to take into account this readjustment of components as soon as equilibrium is disturbed. The toxic action of a toxin-antitoxin mixture would depend, if the physico-chemical concepts apply to such mixtures, not so much on the amount of free toxin present in the solution, as on the amount of available toxin in the mixture. The available toxin includes not only the free toxin, but the toxin that could be formed by a dissociation of the thus-far purely hypothetical toxin-antitoxin compound.

The toxic action of such a mixture can be taken as a measure

of the amount of free toxin present in it only under one of two assumptions. First, that the toxin produces its effects without itself being removed from the solution or in any way altered while in the solution, so that equilibrium is in no way disturbed. This is an unthinkable condition, as a chemical union between cellular substance and toxin is definitely proved. Or, second, it may be taken as a measure on the assumption that the rate of dissociation of the toxin-antitoxin compound is so slow as to give a negligible amount of free toxin during the time occupied by the experiment. This is an improbable assumption, as the guinea-pig experiments usually extend over four or five days.

Similarly, according to the physico-chemical concept, several sera may contain the same amount of "hemolysin,"* but with widely different amounts of free amboceptor and free complement. Here the hemolytic action would depend not only on the amount of "hemolysin" already formed in the sera, but also on the amount of potential "hemolysin" that may be formed from the free amboceptor and free complement present, as soon as equilibrium is disturbed.

The hemolytic power of such a serum can be taken as a measure of the amount of formed "hemolysin" present in it only under one of two assumptions. First, that the "hemolysin" produces its effect without itself entering into chemical union with corpuscles, or in any way being changed, either chemically or in amount, so that the existing equilibrium is in no way disturbed. To say the least, this is an unproved assumption, and almost an unthinkable one. Second, the hemolytic power may be taken as a measure on the assumption that the rate of union of free amboceptor and free complement is so slow as to give a negligible amount of "hemolysin" during the time occupied by the experiment. This assumption is disproved by the fact that artificial amboceptor-complement mixtures have hemolytic powers as soon as made, the hypothetical union taking place with great promptness.

The experimental foundation for the application of physical chemistry to serum pathology rests on the hypothesis that the physiological, chemical, or toxic effect of an unknown solution is a measure

* The term "hemolysin" is used in this paper to designate the hypothetical amboceptor-complement compound in hemolytic serum.

of the amount of a single component present in that solution. If the laws of physical chemistry do govern serum phenomena, this hypothesis is necessarily untrue, as it fails to take into account the readjustment of components as soon as equilibrium is disturbed. Being untrue, there is no way at present discernible by which a single component in such solutions can be measured quantitatively. Consequently measurements thus far made must be regarded as only of historic interest to the physical chemist.

SUMMARY.

1. All attempts to apply physical chemistry to serum pathology have as their foundation the assumption that the physiological, chemical, or toxic effect of certain solutions is a direct measure of the amount of a certain free or bound component in those solutions.
2. This assumption neglects the readjustment of components that the physico-chemical laws themselves necessitate.
3. The assumption, moreover, rests on no experimental grounds, and experimental verification of it is practically out of the question, till toxins, antitoxins, and the like are isolated in a state of purity.
4. Measurements thus far made in serum pathology are therefore only of historic interest to the physical chemist.

THE THIRD SERUM COMPONENT.*

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IN previous papers¹ it has been pointed out that heated hemolytic serum is so changed by exposure to blood corpuscles that direct quantitative comparisons between it and unexposed sera are impossible. This finding is of fundamental importance in serum pathology, as the assumption of the direct quantitative comparability of different sera, toxins, antitoxins, and the like is the basis for all quantitative conclusions thus far drawn in the subject. Work was therefore undertaken to determine the nature of this change, and to discover, if possible, means of overcoming it, and of thus making analyses possible.

This work has led to the conclusion that, in addition to the complement and amboceptor currently assumed as the only active components of hemolytic serum, there is a third component, or series of components, having a marked influence on hemolytic power. Preliminary reports on this third component have already been made.² Although the work is as yet incomplete, it is thought best, at this time, to bring together the data thus far gathered, in order to point the way to the future developments of the subject.

There are two ways in which it is conceivable that exposure of heated hemolytic serum to blood corpuscles may produce qualitative changes in that serum. First, such exposure may change the chemical nature of certain individual serum components. Second, unequal absorption of the different components by the blood corpuscles may bring about disturbances in the quantitative relations, and thus cause a qualitative change in the serum as a whole.

According to current theories there are present in such sera two components: (1) a thermostable amboceptor; and (2) the third

* Work aided by the Rockefeller Institute for Medical Research. Presented before the Chicago Pathological Society April 12, 1906, and before the American Association of Pathologists and Bacteriologists, at Baltimore, Md., May 18, 1906. Received for publication May 27, 1906.

¹ *Jour. Infect. Dis.*, 1905, 2, pp. 485-97; *Trans. Chicago Path. Soc.*, 1905, 6, pp. 319-24; *Jour. of Biol. Chem.*, 1906, 1, pp. 213-18; *Centralbl. f. Bakt.*, 1906, 40, pp. 386-88.

² *Trans. Chicago Path. Soc.*, 1905, 6, pp. 351-58; *Science*, 1906, 23, p. 209; *Jour. Infect. Dis.*, 1906, 3, pp. 225-77.

component, which consists of a mixture of unchanged elements of normal serum, degeneration products of complement ("complementoid"), and hypothetical split products of other thermolabile serum elements. Changes in individual components could not be tested for experimentally till the possibility of there being important changes in quantitative relations is ruled out. Experiments were therefore planned to test the effect on hemolytic power of altering the relative amounts of amboceptor and third component.

To do this various mixtures of amboceptor¹ (heated hemolytic serum containing, of course, its own volume of third component) and third component (heated normal serum) were made, and amboceptor curves² plotted with these mixtures. Two of the simplest curves thus obtained are shown in Fig. 1. Here Curve A represents the changes in hemolytic power as increasing amounts of pure amboceptor are added to a constant amount of complement (normal serum). Curve B shows the changes as increasing amounts of the same amboceptor, plus an equal volume of third component, are added to the same amount of complement. Both curves, of course, are plotted to the same scale, and were made with the same sera, the same corpuscles, and on the same day.

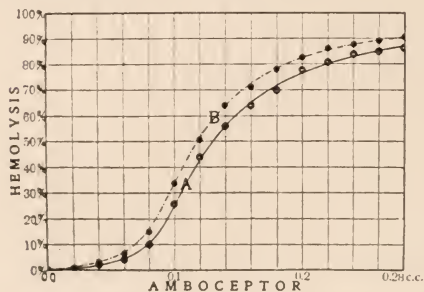


FIG. 1.—Effect of third component on amboceptor curve. A=curve showing changes in hemolytic power as increasing amounts of amboceptor are added to a constant amount of complement (0.25 c.c.). B=Curve showing changes as increasing amounts of the same amboceptor, plus an equal volume of third component, are so added. Curves made with the same sera, same corpuscles, and on the same day.

From these curves it is seen that the addition of third component causes, under the conditions of this particular experiment, an increase in hemolytic power. Less evident is the fact that it also causes a qualitative change in that power, the two sera no longer being analytically comparable.

This qualitative change is most evident if attempts are made to estimate the strength of the new amboceptor in terms of the old. Such an estimation is shown in Table 1, from which

¹ For technic, material, etc., see *Jour. Infect. Dis.*, 1905, 2, p. 461.

² *Ibid.*, 2, p. 471; *Centralbl. J. Bakt.*, 1906, 40, p. 401.

it is seen that the new amboceptor is apparently anywhere from 108 per cent to 120 per cent the strength of the original amboceptor, the percentage depending on the amounts taken for comparison.

TABLE 1.
EFFECT OF THIRD COMPONENT ON APPARENT STRENGTH OF AMBOCEPTOR.
(DATA FROM FIG. 1.)

HEMOLYSIS	AMBOCEPTOR	AMBOCEPTOR PLUS EQUAL VOLUME OF THIRD COM- PONENT	APPARENT STRENGTH	
			Equal Amounts of Amboceptor Compared	Equal Volumes of Total Serum Compared
10%	0.78 c.c.	0.68 c.c.	115%	57.5%
20	0.04	0.84	112	56.0
30	1.04	0.90	108	54.0
40	1.10	1.06	100	54.5
50	1.30	1.18	110	55.0
60	1.48	1.32	112	56.0
70	1.74	1.52	115	57.5
80	2.20	1.80	118	59.0
90	2.80	2.34	120	60.0
Real percentage of amboceptor, 100%			50.0%	

The same phenomenon is seen, in more striking form, in Figs. 2 and 3. The corresponding calculated apparent strengths of amboceptor are shown in Tables 2 and 3.

TABLE 2.
EFFECT OF THIRD COMPONENT ON APPARENT STRENGTH OF AMBOCEPTOR.
(DATA FROM FIG. 2.)

HEMOLYSIS	AMBO- CEPTOR	AMBOCEPTOR PLUS THIRD COMPONENT			APPARENT STRENGTH					
		Half Volume	Equal Volume	Twice Volume	Equal Amounts of Ambo- ceptor Compared			Equal Volumes of Total Serum Compared		
		Curve B	Curve C	Curve D	B	C	D	B	C	D
	c.c.	c.c.	c.c.	c.c.						
10%	0.072	0.064	0.060	0.052	113%	120%	130%	75%	60.0%	45%
20	0.092	0.087	0.085	0.078	105	110	117	72	55.0	39
30	0.102	0.099	0.090	0.091	103	106	112	69	53.0	38
40	0.108	0.106	0.103	0.099	102	105	109	68	52.5	36
50	0.116	0.113	0.110	0.105	103	106	110	69	53.0	37
60	0.122	0.119	0.116	0.111	102	105	110	68	52.5	37
70	0.131	0.127	0.123	0.117	103	107	112	69	53.5	37
80	0.143	0.138	0.132	0.125	105	110	116	70	55.0	39
90	0.170	0.160	0.150	0.140	110	117	126	73	58.5	42
100	0.240	0.208	0.187	0.166	115	128	145	77	64.0	48
Real percentage of amboceptor, 100%					100%	100%	100%	66.3%	50.0%	33.3%

TABLE 3.
EFFECT OF THIRD COMPONENT ON APPARENT STRENGTH OF AMBOCEPTOR.
(DATA FROM FIG. 3.)

HEMOLYSIS	AMBO- CEPTOR	AMBOCEPTOR PLUS THIRD COMPONENT			APPARENT STRENGTH					
		Twice Volume	Seven Times Volume	Seven- teen Times Volume	Equal Amounts of Ambo- ceptor Compared			Equal Volumes of Total Serum Compared		
		Curve B	Curve C	Curve D	B	C	D	B	C	D
10%	c.c.	c.c.	c.c.	c.c.	116%	135%	177%	39%	17%	9.8%
20	0.346	0.296	0.256	0.196	114	124	142	38	15	7.9
30	0.408	0.358	0.330	0.288	111	119	133	37	15	7.3
40	0.452	0.408	0.380	0.338	108	115	128	36	14	7.1
50	0.492	0.456	0.428	0.384	105	112	122	35	14	6.8
60	0.534	0.508	0.476	0.436	103	109	118	34	14	6.5
70	0.576	0.556	0.528	0.488	102	107	114	34	13	6.3
80	0.618	0.608	0.576	0.544	101	106	108	34	13	6.0
90	0.664	0.656	0.602	0.616	102	106	98	34	13	5.4
100	0.734	0.720	0.744	0.744	101	103	91	34	13	5.0
	0.812	0.800	0.788	0.888						
Real percentage of amboceptor, 100%					100%	100%	100%	33 1/3%	12.5%	5.6%

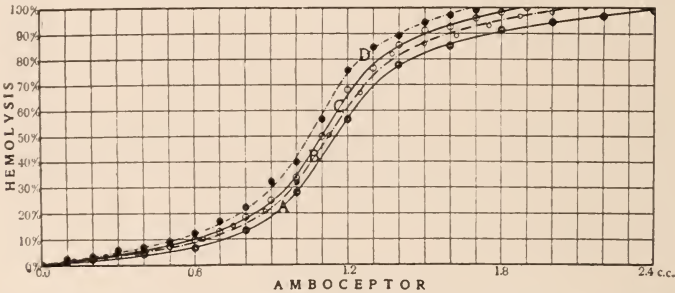


FIG. 2.—Effect of third component on amboceptor curve. A=Curve, as in Fig. 1, showing changes in hemolytic power as increasing amounts of amboceptor are added to a constant amount of complement (0.20 c.c.). B=Curve showing changes as increasing amounts of the same amboceptor, plus half its volume of third component, are so added. C=Curve with an equal volume of third component. D=Curve with two times its volume of third component.

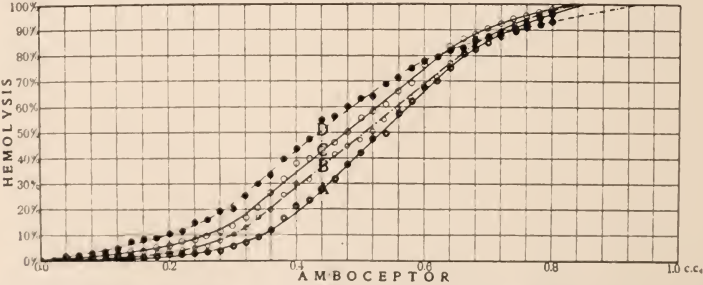


FIG. 3.—Effect of third component on amboceptor curve. A=Curve, as in Figs. 1 and 2, showing changes in hemolytic power as increasing amounts of amboceptor are added to a constant amount of complement (0.22 c.c.). B=Curve showing changes as increasing amounts of the same amboceptor, plus twice its volume of third component, are so added. C=Curve with seven times its volume of third component. D=Curve with 17 times its volume of third component.

In Curve D, Fig. 3, the third component is seen to possess a double power, that of increasing hemolysis when present in comparatively small amounts (maximum 177 per cent), but of decreasing it when present in larger amounts (minimum 91 per cent). This curve strikingly illustrates the fact that changing the relative amounts of amboceptor and third component changes the serum so that it liberates hemoglobin according to a new quantitative law. This change probably accounts for part at least of the change observed in heated hemolytic serum after exposure to corpuscles, as it is inconceivable that all of the serum components could be absorbed equally by the corpuscles. Whether it accounts for the total change in such exposed serum, or not, is still under investigation.

The discovery that under most conditions the third component possesses auxilytic properties (*αὐξάνειν*, to increase) is so at variance with the current belief that "complementoid" lessens serum action by forming inactive amboceptor-"complementoid" compounds, that work was planned to determine, with greater accuracy, the exact rôle of third complement in hemolytic action. To do this, increasing amounts of third component were added to a constant amount of hemolytic serum, and the resulting hemolyses plotted as a curve, showing the changes in hemolytic power as the third component increases.

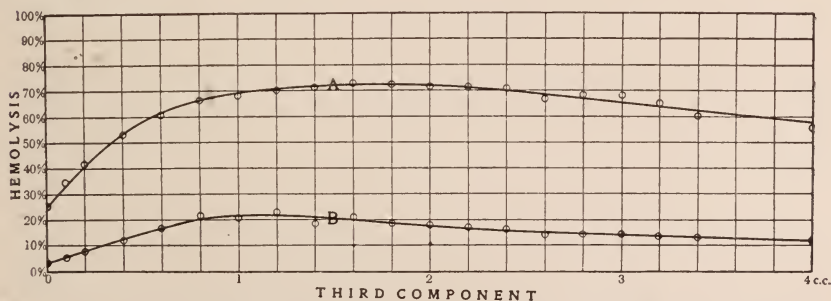


FIG. 4.—Third component curves. Curves showing changes in hemolytic power as increasing amounts of third component are added to constant amounts of hemolytic serum. A=Curve with a constant amount of hemolytic serum capable of producing 26 per cent hemolysis. B=Curve with a constant capable of producing 4 per cent. The curves show an auxilytic action of the third component.

Two curves, obtained in this way, are shown in Fig. 4, and a series of six curves, so obtained, in Fig. 5. Four additional curves are given in Fig. 6. Fig. 6 differs from Figs. 4 and 5, in that, in

place of a constant amount of hemolytic serum, a constant amount of an artificial, hemolytic, ambceptor-complement mixture was used.

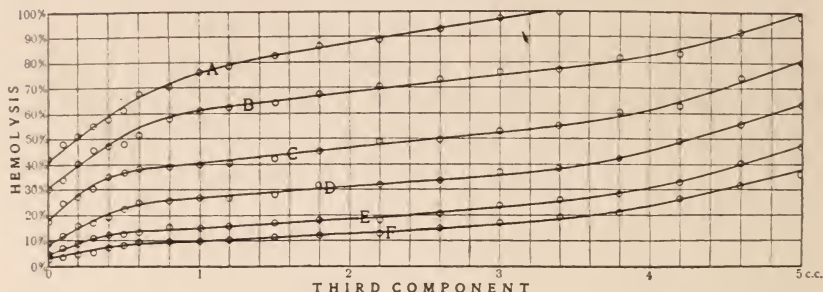


FIG. 5.—Third component curves. Curves, as in Fig. 4, showing changes in hemolytic power as increasing amounts of third component are added to constant amounts of hemolytic serum. The constant amounts of hemolytic serum used in this experiment are capable of producing: 42 per cent hemolysis in Curve A, 31 per cent in Curve B, 18 per cent in Curve C, 8 per cent in Curve D, 5 per cent in Curve E, and 3 per cent in Curve F. The curves show a purely auxilic action of the third component.

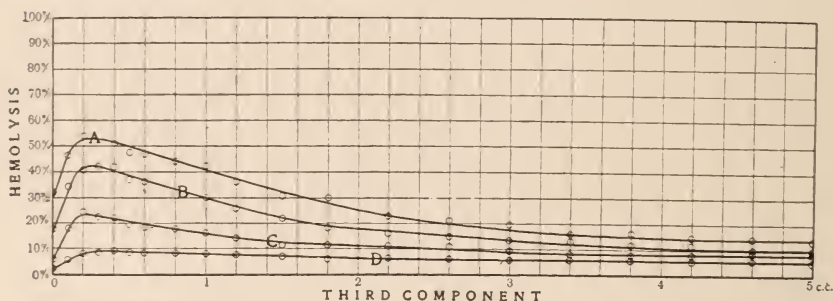


FIG. 6.—Third component curves. Curves, as in Figs. 4 and 5, showing changes in hemolytic power as increasing amounts of third component are added to constant amounts of hemolytic serum. The constant amounts of hemolytic serum used in this experiment are capable of producing: 30 per cent hemolysis in Curve A, 17 per cent in Curve B, 6 per cent in Curve C, and 2.5 per cent in Curve D. The curves show an auxilic action of third component, when used in small quantities; but an antilytic action in larger amounts.

A comparison of these curves is best made by superposing sample curves of each set. Such a superposition is shown in Fig. 7 (Curves B, C, and D). To the figure there have been added four additional curves (A, E, F, and G), taken from subsequent experiments.

From Fig. 7 it is seen that the third component may possess at least four distinct actions: first, it may be practically without effect on hemolysis, as in Curve E; second, it may possess a strong antilytic or inhibiting action on hemolysis, as in Curve G; third, it may

possess a greater or less degree of auxilytic, or increasing action on hemolysis, as in Curves A, B, C, and F; and, fourth, it may possess

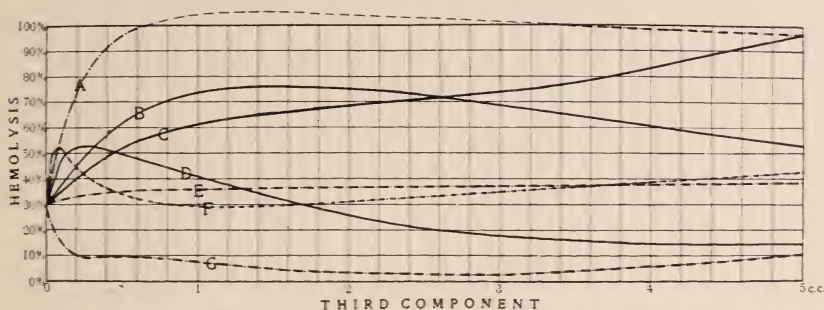


FIG. 7.—Third component curves. Sample curves superimposed from different experiments, showing four different types of third component action.

an auxilytic action when used in small quantities, but an antilytic action in larger amounts, as seen in Curve D. To what are these differences due?

There are two ways in which it is conceivable that the differences in the action of the third complement in different experiments may be brought about. First, it is possible that the sera of different normal animals may differ in the amount or kind of third component they contain. Second, there is the possibility that differences in the treatment of the same serum under different experimental conditions may produce differences in the third component.

To determine whether or not there are differences in the third components of different normal animals, blood was drawn in equal amounts from six normal goats, on the same afternoon, and the sera allowed to separate in the same ice-chest over night. The next forenoon, equal volumes of the sera were measured out into flasks of a uniform size, and the six flasks heated to $56^{\circ}\text{C}.$, in a thermostatic water-bath, such as is used in physico-chemical research, under conditions that assured perfect uniformity of heating. At the end of 60 minutes the flasks were removed from the water-bath, and cooled by immersing in ice-water. Curves were then plotted with the resulting third components.

The six curves obtained in this way are shown in Fig. 8. Five additional curves, obtained in the same way, but with the third components heated to $59^{\circ}\text{C}.$, for 60 minutes, are shown in Fig. 9.

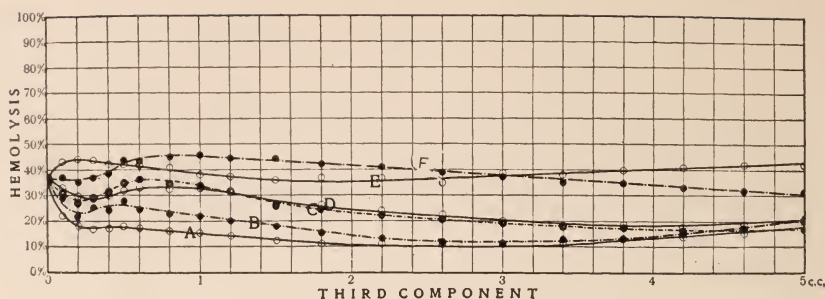


FIG. 8.—Comparison of third components of different normal sera. Curves showing changes in hemolytic power as increasing amounts of third component from six different normal animals are added to the same constant amount of hemolytic serum. Third components heated to 56° C. for 60 minutes.

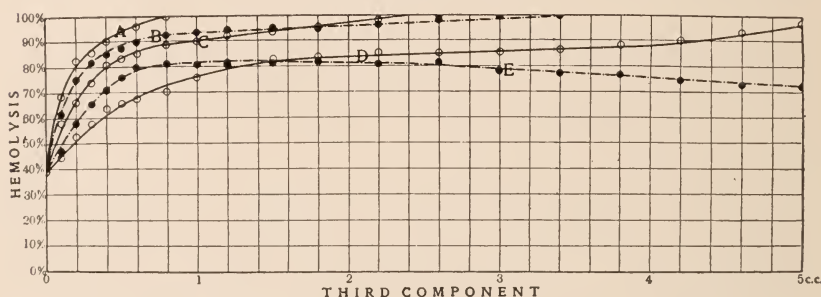


FIG. 9.—Comparison of third components of different normal sera. Curves showing changes in hemolytic power as increasing amounts of third component from five different normal animals are added to the same constant amounts of hemolytic serum. Third components heated to 59° C. for 60 minutes.

From Fig. 8 it is seen that no two of the six normal animals that were tested have sera that yield the same third component, under identical conditions. In four of the animals, the third component was to a greater or less degree antilytic (Curves A, B, C, and D), while in the other two it was, in certain amounts, slightly auxilytic (Curves E and F). In Fig. 9, while all the third components are strongly auxilytic, the differences between them are equally striking.

This finding is of broad biological and medical significance. Its main interest, however, in the present paper lies in its bearing on quantitative serum investigations. Quantitative measurements with hemolytic sera will be out of the question, till a method is devised by which the action of the third component can either be entirely eliminated, or, at least, made uniform in different sera. Two sera, possessing identical amboceptor and complement, may have widely

different hemolytic action, if the the third components of one is auxilic, while that of the other is antilytic.

To test the second hypothesis, that differences in experimental conditions may produce differences in third component, serum was drawn in a large amount from one normal animal, or a large amount of carefully mixed sera from two or more animals was used, and equal volumes of this serum heated, at a uniform temperature, for different periods of time. Curves were then plotted with the resulting third components.

Five curves, obtained in this way, are shown in Fig. 10. Additional curves, so obtained, are given in Figs. 11 and 12.

From these curves it is seen that differences in the length of time of heating the serum may produce marked differences in the third component. In each set of curves, heating for more than 30 minutes

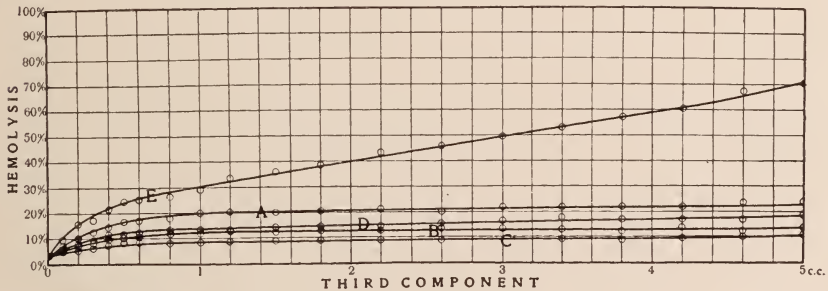


FIG. 10.—Effect of prolonged heating on the third component. Third component curves, as in Fig. 8, but with the same normal serum heated for different periods of time. A=Curve with serum heated to 56° C., for 30 minutes; B=Curve with serum heated 60 minutes; C=heated 100 minutes; D=150 minutes, and E=220 minutes.

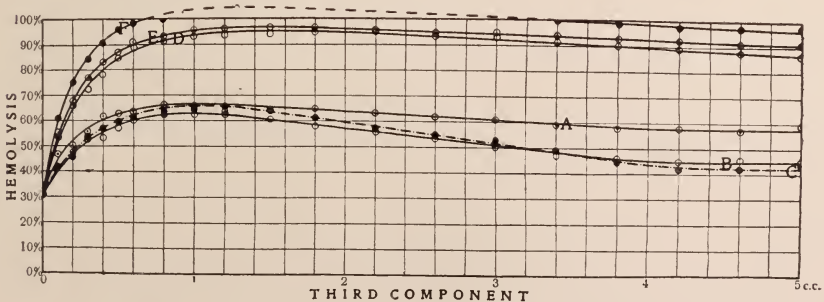


FIG. 11.—Effect of prolonged heating on the third component. Third component curves, as in Fig. 10, with the same normal serum heated to 56° for different periods of time. A=Curve with serum heated for 30 minutes; B, for 45 minutes; C, for 60 minutes; D, for 100 minutes; E for 130 minutes, and F, for 165 minutes.

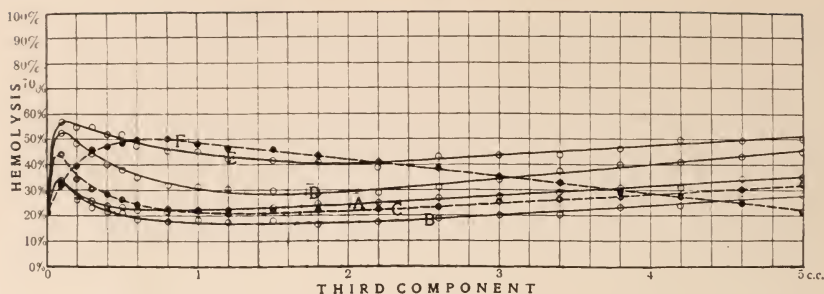


FIG. 12—Effect of prolonged heating on third component. Third component curves, as in Figs. 10 and 11, with the same normal serum, heated to $56^{\circ}\text{C}.$, for different periods of time. Time of heating: Curve A=35 minutes, Curve B=65 minutes, Curve C=108 minutes, Curve D=165 minutes, Curve E=240 minutes, Curve F=330 minutes.

caused a distinct drop in the third component curve. This drop was succeeded by a marked rise when the serum was heated for more than 60 minutes. In Fig. 12, this rise was succeeded by a second fall and by a marked change in the nature of the curve, when the heating was prolonged beyond four hours.

In order to follow these changes in greater detail, a flask of normal serum was heated at a uniform temperature for 10 hours, and an accurately measured amount of serum removed, at 10-, 20-, and 30-minute intervals throughout that time, and tested for its effect on hemolytic power.

A curve obtained in this way, by heating the serum to $56^{\circ}\text{C}.$, is shown in Fig. 13. Six curves of the same nature, obtained by using different constant amounts of third component, but the same

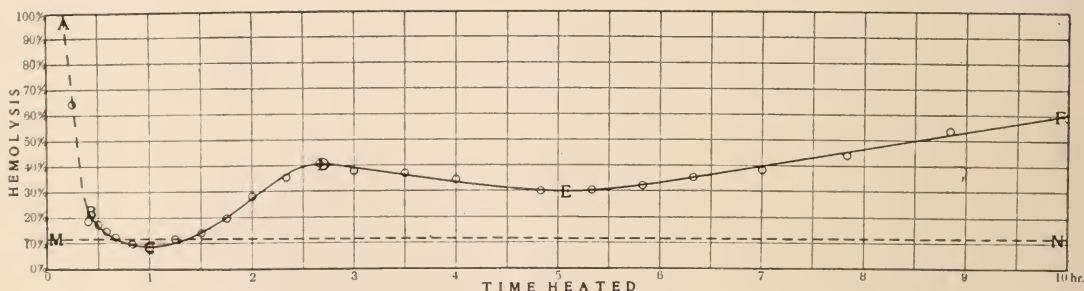


FIG. 13—Effect of prolonged heating on third component. Curve showing changes in auxilic and antilytic properties of third component, when heated to $56^{\circ}\text{C}.$ for 10 hours. Curve at each stage of the heating shows the effect on hemolytic power of adding a constant amount of third component (1.8 c.c.) to a constant amount of hemolytic serum. The hemolytic serum in itself, is capable of producing 12 per cent hemolysis (MN). Dotted portion of curve (AB)=curve before the complete destruction of complement. This curve is the average of the six curves shown in Fig. 14.

constant amount of hemolytic serum, are shown in Fig. 14. A curve obtained in the same way, but with serum heated 59°C ., is shown in Fig. 15, and six parallel curves, so obtained, in Fig. 16.

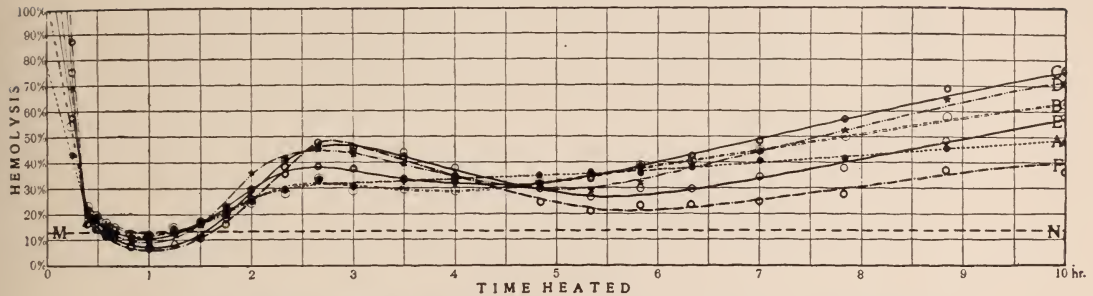


FIG. 14—Effect of prolonged heating on third component. Curve as in Fig. 13, showing changes in auxilic and antilytic properties of third component, when heated to 56°C . for 10 hours. Constant amount of hemolytic serum used in this experiment is capable of producing 12 per cent hemolysis (MN). The constant amounts of third component used in each curve are: Curve A=0.25 c.c., Curve B=0.5 c.c., Curve C=1 c.c., Curve D=2 c.c., Curve E=3 c.c., and Curve F=4 c.c.

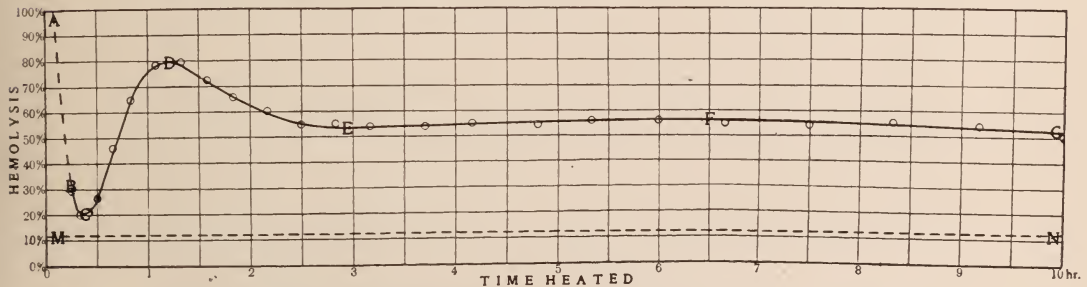


FIG. 15—Effect of prolonged heating on third component. Curve, as in Fig. 13, showing changes in auxilic properties of third component, when heated to 59°C . for 10 hours. This curve is the average of the six curves in Fig. 16. Constant hemolytic serum is capable of producing 13 per cent hemolysis (MN). Dotted portion (AB) is curve before the complete destruction of complement.

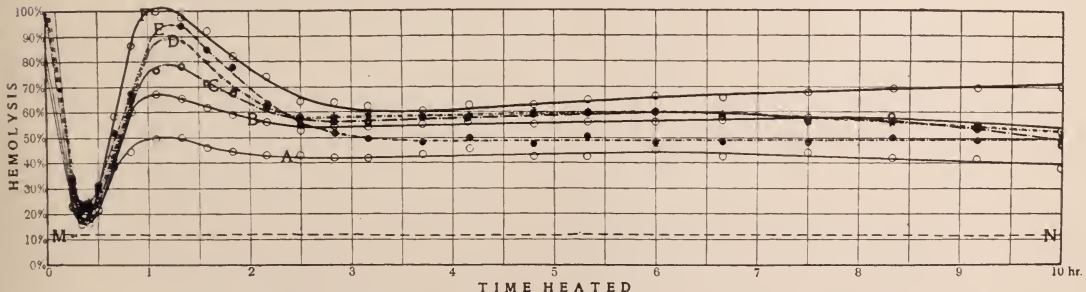


FIG. 16—Effect of prolonged heating on third component. Curve, as in Fig. 14, showing changes in auxilic properties of third component when heated to 59°C . for 10 hours. The constant amounts of third component used in each curve are: Curve A=0.3 c.c., Curve B=0.6 c.c., Curve C=1 c.c., Curve D=1.5 c.c., Curve E=3 c.c., Curve F=5 c.c. Constant hemolytic seen capable of producing 13 per cent hemolysis (MN).

From these curves it is seen that, when serum is heated to 56°C ., complete destruction of complement takes place in about 20 minutes. At that time the third component possesses a slight auxilytic action (primary auxilysin possibly due to a trace of undestroyed complement, though complement could not be detected experimentally). As the heating is continued beyond 20 minutes there is a diminution in this auxilytic power. The power completely disappears at the end of about 40 minutes' heating and is succeeded by an antilytic power. This antilytic action reaches a maximum in about 60 minutes, when it in turn decreases and entirely disappears in about 110 minutes.

On prolonging the heating beyond 110 minutes, there is produced a second auxilytic power (secondary auxilysin), which reaches a maximum in about three hours' heating. This secondary auxilysin in turn decreases, reaching a minimum at the end of about five hours. After this there is a gradual increase in auxilytic power (tertiary auxilysin), which apparently has not reached its maximum, at the end of 10 hours' heating.

When the serum is heated to 59°C ., the same general phenomenon takes place, except that the production of the antilytic substance is then apparently masked by the more marked or more rapid production of the secondary auxilysin, and the destruction of the secondary auxilytic substance, is apparently modified by the more rapid production of the tertiary auxilysin. The tertiary auxilysin reaches a maximum in about six hours, after which there is a slight decrease in auxilytic power.

Changes in the nature of the third component curve during prolonged heating can be determined roughly from Figs. 14 and 16. Cross-sections of Fig. 14 at different periods of time give the third component curves shown in Fig. 17. Cross-sections of Fig. 16 give the curves shown in Fig. 18. Figs. 17 and 18 show the same general changes in the third component curve previously noted, except that here by taking the cross-sections at selected times, the observed changes are more pronounced.

It was shown in the fore part of the paper that there are differences between the third components of different normal animals, prepared under identical conditions. What is the cause of this difference?

It is conceivable that the difference is due either to differences in the amounts of the various auxilytic and antilytic substances

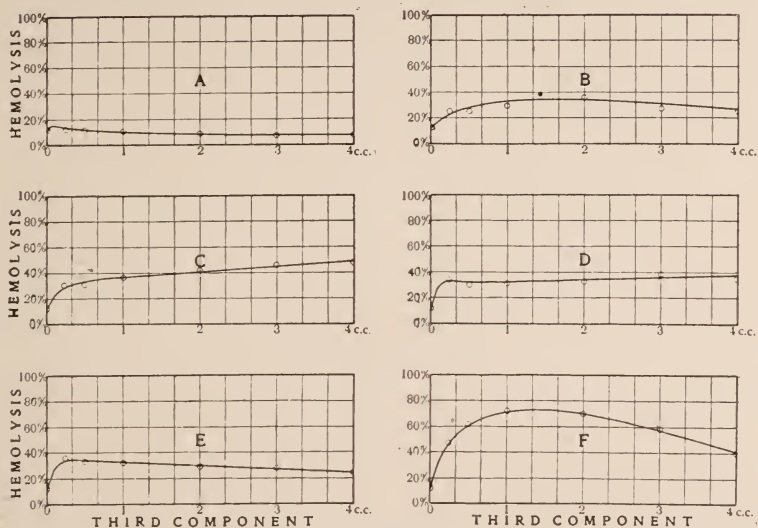


Fig. 17.—Changes in third component curve during prolonged heating. Curves drawn from data in Fig. 14. Length of heating: Curve A=60 minutes, Curve B=120 minutes, Curve C=180 minutes, Curve D=240 minutes, Curve E=300 minutes, Curve F=600 minutes. Temperature=56° C.

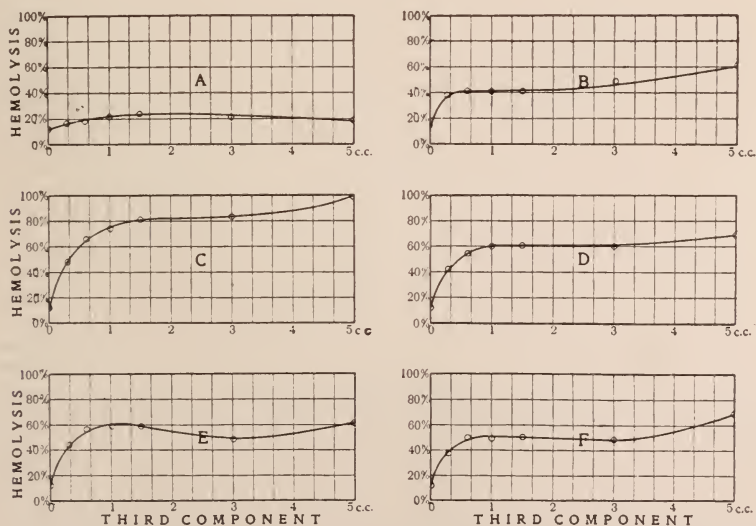


Fig. 18.—Changes in third component curve during prolonged heating. Curve drawn from data in Fig. 16. Length of heating: Curve A=20 minutes, Curve B=40 minutes, Curve C=60 minutes, Curve D=140 minutes, Curve E=240 minutes, Curve F=600 minutes. Temperature=59° C.

formed in these sera during prolonged heating, or to differences in the times at which these substances are so formed. To test these hypotheses, different normal sera were heated together in the same thermostatic water-bath, and parallel curves plotted, showing the changes in the auxilytic and antilytic properties of each serum during prolonged heating.

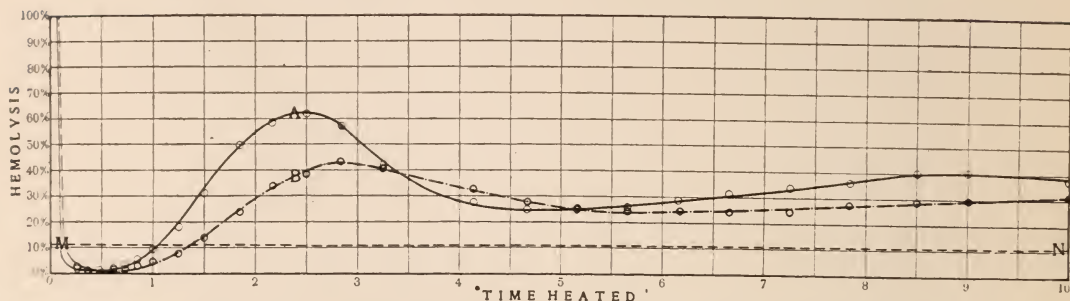


FIG. 19.—Comparison of different third components during prolonged heating. Curves, as in Fig. 13, showing changes in auxilytic and antilytic properties of the third components of two different normal animals, when the sera are heated to 56° C. for 10 hours. The constant amount of hemolytic serum used in the experiment is capable of producing 11 per cent hemolysis (MN). Curve A is the average of Curves C and D of Fig. 20, and Curve B, the average of Curves A and B of the same figure.

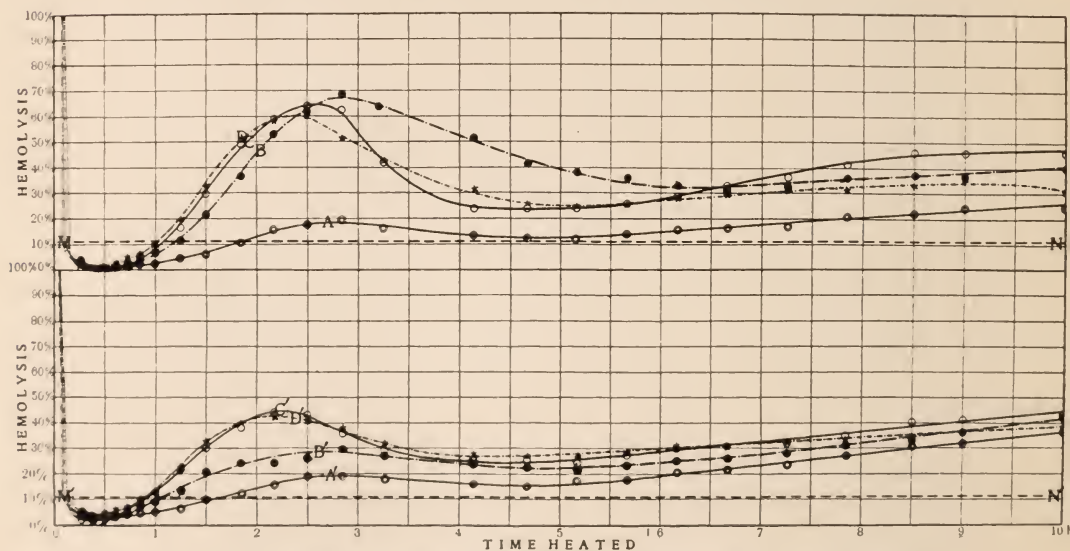


FIG. 20.—Comparison of different third components during prolonged heating. Curves, as in Fig. 13, showing changes in auxilytic and antilytic properties of the third components of the four different normal animals, when their sera are heated to 56° C. for 10 hours. The constant amount of third component used in the upper set of curves (A, B, C, and D)=3 c.c., in the lower set (A', B', C', and D')=0.5 c.c. Constant hemolytic serum used in both sets is capable of producing 11 per cent hemolysis (MN, M'N').

Two curves, obtained in this way, are shown in Fig. 19. Two sets of four curves each, so obtained, are given in Fig. 20.

From Figs. 19 and 20 it is seen that the difference observed between the third components of different normal sera, is due partly to slight differences in the times at which the various auxilytic and antilytic substances are produced or destroyed; but mainly due to differences in the amounts of these substances so produced. Thus, Curve A, of Fig. 20, shows the production of a very small amount of secondary auxilysin, while Curves B, C, and D show comparatively large amounts of that substance.

It should be noted, in concluding the presentation of this data, that at no time in any of the above experiments was the third component found to possess independent hemolytic powers, and at no time was it capable of sensitizing corpuscles or of reactivating amboceptor. The auxilysins have, in all cases, been incapable of acting, except in the presence of both amboceptor and complement.

No theory is as yet advanced as to the nature of the various auxilytic and antilytic substances herein reported, nor as to the bearing of this report on the fundamental concepts of serum pathology.

SUMMARY.

1. Normal goat serum in which the complement has been completely destroyed by heat is capable of exerting considerable influence on hemolytic action, when tested with a goat serum immunized against sheep corpuscles.

2. The action of the third serum component or the substance remaining after such heating, varies greatly in different experiments. In one experiment it may be purely auxilytic; in another, purely antilytic; in a third, auxilytic in certain amounts and antilytic in others; and in a fourth, practically inactive.

3. Sera of different normal animals, heated under identical conditions, may differ widely in the action of their third components.

4. Differences in the length of heating and in the temperature at which it is heated, produce marked differences in the third component of the same normal serum.

5. Normal serum, heated in bulk to 56° C., loses its complement in about 20 minutes. On prolonging the heating there are

formed in the order in which they are mentioned: (1) a primary auxilysin, (2) a primary antilysin, (3) a secondary auxilysin, and (4) a tertiary auxilysin. Each of these substances appears, and afterward in part or wholly disappears, at a definite time during the heating.

6. When heated to 59° C., the same general phenomenon takes place, except that the events occur with greater rapidity.

7. Different normal sera, heated under identical conditions, differ but slightly in the times at which the various antilytic and auxilytic substances are produced and destroyed. They differ, however, greatly, in the amount of each substance so formed.

8. At no time during prolonged heating, does the third component acquire independent hemolytic powers, and at no time does it become capable of giving hemolytic properties to pure component or to pure amboceptor.

9. No theories are as yet advanced as to the nature of the auxilytic and antilytic substances herein reported, nor as to the bearing of the above facts on fundamental theories of serum pathology.

THE CAUSE OF GREEN COLORATION OF BACTERIAL COLONIES IN BLOOD-AGAR PLATES.*

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(From the Memorial Institute for Infectious Diseases, Chicago.)

IN 1903 Schottmüller,¹ and very soon afterward Rosenow,² showed that pneumococci produce green colonies in blood-agar plates, while *Streptococcus pyogenes* produces small, grayish, hemolyzing colonies. Schottmüller showed that there are streptococci which produce green colonies in these plates very similar to the pneumococcus colonies. I have found pneumococci which produce hemolyzing colonies similar to those of *Strept. pyogenes*.

In making use of blood-agar plates for the purpose of studying the streptococci and diplococci found in throats, I have noticed a number of very interesting peculiarities of some cultures. A small number of strains of streptococci were found which produce the characteristic hemolyzing colonies in plates made of plain agar and 0.4 c.c. of blood, and green colonies of the pneumococcus type in plates made of glucose agar and blood. Furthermore, it was found that a large number of typical *Strept. pyogenes* cultures produce no hemolysis in plates made of glucose blood-agar, although they produce very extensive hemolysis in plates made of plain agar and blood. If these glucose blood-agar plates are allowed to remain in the incubator for 36 to 48 hours, the streptococcus colonies become distinctly green and are surrounded by a green halo. In some instances the green coloration is observed at the end of 24 hours.

These facts led me to inquire into the cause of the green coloration, and the idea presented itself that it is in some way connected with the production of acid by the colonies. This idea was then tested, and all the facts which have been ascertained point to the correctness of the view. Sugar-free agar was prepared by dissolving 0.5 per cent NaCl, 1 per cent peptone (Witte), and 1.5 per cent agar-agar in distilled water, clarifying, tubing, and sterilizing in the autoclav.

* Received for publication June 1, 1906.

¹ *Munch. med. Wchnschr.*, 1903, 50, pp. 849, 909.

² *Jour. Infect. Dis.*, 1904, 1, p. 308.

It was found now that pneumococci which produce distinctly green colonies in ordinary plain blood-agar, or glucose blood-agar plates do not produce more than a faint trace of green in plates composed of sugar-free agar and 0.3 to 0.4 c.c. of fresh defibrinated rabbit blood. But if 1 per cent of glucose, lactose, or inulin is added to the sugar-free agar, these cultures again produce deep green colonies in blood-agar plates made with it. It is necessary that the cultures should rapidly ferment inulin if they are to produce green colonies in inulin blood-agar plates.

On the other hand, it has been found that *Strept. pyogenes*, which does not readily ferment the polysaccharides, produces hemolyzing colonies in inulin, dextrin, and usually in lactose blood-agar plates, although it forms green colonies in plates of glucose blood-agar.

Several other cultures were tested, and it was found that *Staph. aureus* and *B. typhosus* produce distinctly green colonies in glucose blood-agar plates, although there is no trace of green in the plates made of plain agar and blood. *B. coli communis* was also tested, and it was found that this organism produces rapid and extensive hemolysis in all blood plates, and hence no green coloration could be detected.

It might be asked now why *Strept. pyogenes* does not produce green colonies in ordinary plain blood-agar plates. This is probably due to two reasons: (1) these organisms probably do not readily ferment the muscle sugar which is found in plain agar, and (2) they produce such rapid hemolysis that the green would not be detected, because we can see no green coloration after the red corpuscles have been completely hemolyzed.

It is quite certain from these experiments that the green coloration of bacterial colonies in blood-agar plates is dependent upon the production of acid. As all the cultures which have been worked with produce lactic acid in glucose broth (as shown by the ferric chloride test), it is very probable that the green color is caused by the action of lactic acid on the red corpuscles or on the hemoglobin. It cannot yet be stated whether the green color is due to a blending of colors or to the production of a pigment, possibly biliverdin. Further work must be done on this point.

It must be mentioned that pneumococcus colonies produce dif-

ferent degrees of green coloration in plates made of sugar-free agar and different kinds of blood. Whereas there may be no trace of green with one sample of blood, in plates containing blood from a different animal the colonies may have a decided greenish tint. This may possibly be due to the slight variation in the sugar content of the blood.

CONCLUSIONS.

The green coloration of bacterial colonies in blood-agar plates is dependent upon the production of acid and the action of this acid (probably lactic acid) on the red corpuscles.

B. coli communis produces extensive hemolysis in blood-agar plates.

GENERALIZED BLASTOMYCOSIS.

REPORT OF A CASE WITH MILIARY AND ULCERATIVE BLASTOMYCOSIS
OF THE LUNGS; MILIARY BLASTOMYCOSIS OF THE SPLEEN;
AND MULTIPLE SUPERFICIAL AND DEEP ABSCESES.*

E. E. IRONS AND E. A. GRAHAM.

(From the Surgical Clinic of Dr. D. W. Graham and the Pathological Laboratory, Rush Medical College,
Chicago.)

It is now known that infection with certain members of the group *Oidium* may become disseminated throughout the human body, and *generalized blastomycosis* or *oidiomycosis* must be recognized as a clinical entity. Several cases have been reported, including five from Chicago, but until recently certain observers, particularly among the Germans, have been inclined to doubt the statement that blastomyces was the sole etiological factor in some at least of the cases reported. The present case † presents several points of clinical and pathological interest, and in reporting it somewhat at length it is hoped that something may be added to our knowledge of the clinical course and pathology of the disease.¹

THE CLINICAL HISTORY.

A. D., age 47, German, lumberman. Admitted to the Presbyterian Hospital September 11, 1905.

The family history is negative as to tuberculosis and malignant diseases. A wife and two children are living and well; no history of miscarriages. The patient had typhoid fever 14 years ago. Otherwise has had no severe illness until the present. Denies all venereal infection. Has never suffered from sore throat, alopecia, nor eruption of any kind until the present lesions appeared. Has taken alcohol in small amounts. Has lived in Chicago for a number of years working in a lumberyard until obliged to give up work on account of the present trouble.

In March, 1905, the patient noticed a small subcutaneous nodule on the inner surface of the right thigh about five inches below Ponpart's ligament. This nodule was slightly tender, and gradually increased in size to that of a small hen's egg, softened, and finally broke through the skin with the discharge of bloody pus, leaving an ulcer

* Received for publication June 1, 1906.

† The case was demonstrated from time to time in the clinic by Doctor Graham, to whom we are indebted for the opportunity of studying the clinical course of the disease.

¹ For bibliographies reference may be made to the following articles: RICKETTS, *Jour. Med. Res.*, 1901, 1, p. 373; BUSCHKE, *Bibliotheca Medica*, Stuttgart, 1902, Abtheil. 2, Heft 10; BASSOE, *Jour. Infect. Dis.*, 1906, 3, p. 91.

which healed slowly with the formation of a slightly indurated reddish-brown scar. Previous to the appearance of this lesion there had been no cutaneous lesions on any part of the body.

Before the first lesion on the thigh had healed a second appeared on the forehead and then other similar lesions on the legs, hips, and arms, in rapid succession, all of which ran a course similar to that of the first, unless incised, in which case healing was slightly more rapid.

The constitutional symptoms which were relatively slight at first, became more marked, and about six weeks after the onset, the patient was confined to his room, largely on account of weakness, but undoubtedly partly by reason of the unsightly appearance of the lesions on his face and arms, in regard to which he was very sensitive, having been told by his physician that they were of syphilitic origin.

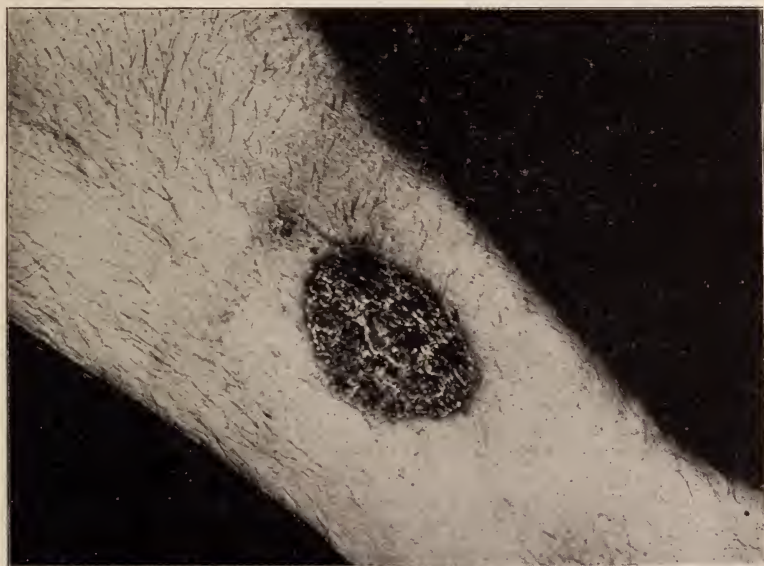


FIG. 1.—Old, partially healed ulcer of the leg present on admission to the hospital.

The chief symptoms at this time were weakness, anorexia, and slight fever. Previous to admission to the hospital there was no cough or other pulmonary difficulty. Physicians who had seen him up to this time were divided in their diagnoses between syphilis and tuberculosis.

Two months after the appearance of the first lesion, the right and then the left ankle became swollen, painful, and slightly reddened. Lesions over the malleoli similar to the others appeared soon after, but they did not heal so rapidly, and on admission to the hospital six months after the appearance of the first nodule on the thigh, both ankles were considerably swollen, red, and tender, so that it was almost impossible for the patient to stand, on account of the pain produced.

Examination on admission.—The patient is a middle-aged man, medium height,

rather poorly nourished, and shows a marked anemia. There are several slightly indurated scars over the forehead. The eyes, ears, and nose are normal; tongue coated; pharynx normal. There are several hard pea-sized glands in the posterior triangles of the neck. Heart and lungs are negative. Abdomen and genitalia normal. At various points over the body are lesions in all stages of development.

Scars of healed and partially healed lesions over the forehead, right forearm, both hips, thighs, and legs (Fig. 1). Fluctuating abscesses over both elbows (Fig. 2), left forearm, left patella, right heel, left external malleolus. Both ankles are swollen, painful, tender on pressure, dull red in color, and slightly edematous. There is some limitation of motion, though this seems to be due largely to periarticular swelling and tenderness.



FIG. 2.—Subcutaneous abscess which has discharged spontaneously by erosion of the overlying skin.

The more superficial lesions begin as small, hard, subcutaneous nodules, gradually enlarging, with some tenderness. They soon become soft, fluctuate, and, if not incised, break through the skin with the discharge of reddish bloody pus in which can be demonstrated yeastlike, budding organisms, often in immense numbers. Other abscesses are deeply seated, some of them being subperiosteal in origin. In the deeper lesions there is a tendency to extensive dissection along the intermuscular fasciæ after the manner of tuberculous abscesses.

The superficial lesions present on admission, said by the patient to have followed abscesses, present a roughly oval outline, edges fairly regular and slightly raised, with occasional though rare miliary abscesses, such as are found in the ordinary lesions of blastomycetic dermatitis. In none of these miliary abscesses is it possible to demonstrate the organisms. Surrounding each ulcer is a dark red, often purplish zone. The ulcer has a rather irregular floor formed by granulation tissue, with here and there processes of newly-formed epidermis extending inward from the margins. The

whole is covered by a dry, rather firmly attached, horny crust. The ulcers show no tendency to progress, and the healing though slow is continuous. The scars are reddish-brown, occasionally indurated, but in general slight in comparison with the extent of the original lesions.

Those ulcers which followed abscesses developing while in the hospital showed several points of difference from the foregoing, due to the treatment. Instead of being allowed to enlarge until spontaneous evacuation by erosion of the skin occurred, the abscesses were incised and the pus evacuated as soon as fluctuation could be demonstrated. The initial damage to the skin and subcutaneous tissue was thus much decreased, and the lesions remained as freely discharging superficial abscesses, which healed fairly rapidly up to the last few months of illness, when there was evident a tendency to sloughing of the subcutaneous tissue with undermining of the skin.

Course.—After a few days' rest in bed the swelling and redness of the ankles decreased. Two abscesses over the malleoli were drained, and denuded bone found. The patient was allowed up in a wheel chair, which he used almost daily up to the day of his death.

The lungs at the time of admission (September 11) were normal on physical examination. There was no cough or other pulmonary symptoms. On September 25 the patient for the first time had a slight cough and complained of some pain in the left side. Examination showed slight dulness and harsh breath sounds, together with a few fine moist râles in the left infraclavicular and midscapular regions. No friction could be demonstrated. The remaining chest area was normal, and there was no coincident change in constitutional symptoms. The expectoration was small in amount and mucopurulent. Repeated examinations failed to show blastomycetes or tubercle bacilli.

Two weeks later the cough and pain had disappeared and aside from slight relative dulness in the left midscapular region, the chest findings were normal.

On November 6 the general condition of the patient was less favorable. The cough and pain in the chest reappeared. Examination showed dulness and râles in both infraclavicular fossæ. Posteriorly in the midscapular region on both sides were signs of consolidation, more marked on the left side. The expectoration was mucopurulent, moderate in amount, and on examination showed large numbers of blastomycetes. These were easily demonstrated in fresh specimens of sputum mounted in 1 per cent potassium hydrate, and also in coverslip preparations stained with methylene blue, and hematoxylin and eosin (Fig. 3). No mycelial threads were found. The sputum was again repeatedly examined for tubercle bacilli, with negative results. The relative absence of secondary infection was a noticeable characteristic of the sputum at this time, which consisted almost entirely of mucus, leucocytes, epithelial cells, and the budding organisms. With the extension of the disease in the lungs the sputum increased in quantity to about 125 c.c. in 24 hours, and occasionally small amounts of blood appeared intimately mixed with the mucopurulent secretion. Active hemorrhage did not occur.

From this time on the patient grew steadily weaker. New lesions appeared in rapid succession over the body, particularly involving the extremities and head, and with the decline in general condition healed correspondingly more slowly. At several points including the left malar and left frontal bones, right patella, and right external malleolus denuded bone could be demonstrated.

During the last three weeks of life there was marked destruction of the subcutaneous

tissue at the sites of the lesions, so that relatively large areas of skin were undermined in spite of the employment of free drainage. A large abscess which formed over the right hip sloughed extensively a few days before death. There were no signs or symptoms of invasion of the vertebræ. On the day of death the patient became rapidly worse, with some elevation of temperature, rapid pulse, and delirium, presumably



FIG. 3.—Sputum containing blastomycetes. Coverglass preparation. Hematoxylin and eosin. Photomicrograph, $\times 1,500$.

due to an intercurrent terminal infection. Death occurred on January 18, 1906, 10 months after the appearance of the first lesion.

The temperature while the patient was under observation in the hospital was irregular, often normal in the morning and rising to 101° to 102° in the evening. At other times it remained from 99° to 100° for several days at a time. Frequently the increase in temperature was coincident with the appearance of a new abscess, though this was not always the case. The elevations of temperature were not accompanied by chills. The pulse showed no unusual characteristics, varying with the temperature,

though during the last three months of illness the average pulse-rate was relatively increased.

Blood.—On admission (September 11) the blood count showed 16,000 leucocytes per c.mm.

On October 15 a blood examination showed the following:

Erythrocytes	4,320,000
Leucocytes	12,500
Hemoglobin (Dare)	45%

A differential count of 250 leucocytes showed—

Polynuclear neutrophiles.	75.6%
Large mononuclears	7.6
Small mononuclears	10.8
Indentate nucleus	2.0
Eosinophiles	3.2
Myelocytes	0.8

The red cells were pale and averaged rather smaller in size than normal. No nucleated forms were found. There was slight polychromasia present.

A third blood examination on December 15 gave the following:

Erythrocytes	4,160,000
Leucocytes	21,200
Hemoglobin (v Fleishl)	55%

At this time the patient was markedly emaciated and more anemic in appearance than on admission. There was also a secondary staphylococcus infection of two abscesses when this count was made.

A differential leucocyte count showed—

Polynuclear neutrophiles	93.2%
Large mononuclears	3.2
Small mononuclears	2.8
Eosinophiles	0.8

There were slight poikilocytosis and polychromasia, but no nucleated reds were found.

Blood cultures.—Blood cultures were made on two occasions (December 15 and December 30), using various media including blood serum, glycerin, glucose-agar slants, broth tubes and flasks, and glucose-agar plates. The cultures were sterile throughout.

Urine.—The urine on admission was yellow, clear, acid, and showed a trace of albumen; no sugar. A few hyalin and finely granular casts were found in the centrifugized specimen. A 24-hour specimen of 1,625 c.c. had a specific gravity of 1.015 with a trace of serum albumen; no sugar. In the sediment were occasional hyalin and finely granular casts and a few leucocytes. Several subsequent examinations up to within a few days of death gave practically the same results. At no time were blastomycetes found in the urine, although careful search was repeatedly made in view of the renal involvement reported in some other cases of generalized blastomycosis.

Feces.—The bowels were practically normal throughout the illness. There was no diarrhea and the feces were normal in appearance.

Treatment.—The abscesses as they appeared were incised and drained, with subsequent daily dressings. To some of the abscesses a 1 per cent aqueous solution of copper sulphate was applied, while others were treated with plain sterile gauze.

During the first month of this treatment, the lesions on which the copper sulphate was used healed somewhat more rapidly than the others, but owing to the rapid decline in the general condition of the patient from this time on, any further comparison of the methods would be only misleading. The copper sulphate did, however, materially reduce the tendency to secondary infection, and it was subsequently used on all abscesses. It is worthy of note that with numerous lesions over all the extremities, the dressings of which occasionally were unavoidably displaced by the movements of the patient, there was practically no infection with ordinary pyogenic organisms. Certain of the superficial lesions present on admission were treated with the X ray with no marked benefit.

Internally in addition to full diet and general tonic treatment, the patient was given potassium iodide in increasing doses up to 60 grains per day. For a time also he received copper sulphate gr. $\frac{1}{2}$ three times a day, but after a short time this was discontinued on account of gastrointestinal disturbance.

Cultural and animal experiments with materials obtained during life.—The pus from the abscesses was of a bloody, fluid character, consisting of blood cells, pus cells, tissue debris, and the organisms, often in large numbers. Only the yeastlike forms were found in the pus. These were easily demonstrated in the fresh preparations mounted in 1 per cent potassium hydrate.

Although the organisms occurred in pure culture and often in large numbers in the abscesses, considerable difficulty was experienced in obtaining growths on artificial media. For example, of six complete series of cultures on various media, including broth, glucose agar, glycerin agar, and blood serum, in only two series were growths obtained, and in these the growths occurred on practically all the media employed. Inasmuch as there were no recognizable differences in the media of the several series, it would appear that certain variations occur from time to time in the ability of the organisms to withstand the change of environment entailed in the transfer from the tissues to artificial media. Having once obtained growths on artificial media, no difficulty was experienced in maintaining them.

The organisms were obtained in pure culture from abscesses on the forearm and on the patella, and also on one occasion from the sputum.

With a view to excluding the tubercle bacillus as a possible factor in the disease, pus from the abscesses was inoculated intraperitoneally and subcutaneously into guinea-pigs, and the animals examined from three to six weeks later. No trace of tuberculous infection could be found in any of these animals. As already stated, many preparations from the pus and sputum were stained for tubercle bacilli with negative results.

THE NECROPSY RECORD.

The necropsy was performed six hours after death by Dr. E. R. LeCount.

Anatomical diagnosis.—Miliary blastomycosis of lungs and spleen; ulcerative blastomycosis of the upper lobe of the left lung; multiple subcutaneous abscesses and sinuses involving the face, scalp, and all the extremities; retroesophageal abscess with erosion of the bodies of the seventh cervical to the fifth dorsal vertebræ (inclusive) and the anterior surfaces of the vertebral extremities of the second to the fifth left ribs; erosion of left parietal bone; sloughing deep ulcer of right thigh; abscess of thyroid cartilage; subpleural hemorrhages of right lung; bilateral fibrous pleuritis; hyperplastic splenitis; hyperplasia of mesenteric lymph glands; brown atrophy of the heart; colloid goiter (all lobes); slight sclerosis of anterior mitral leaflet and root of

aorta; chronic catarrhal gastritis; localized fibrous peritonitis; chylous ascites (slight); slight atrophy of liver; retention cysts of left kidney.

The body is that of a middle-aged, much emaciated man. Body still warm. There are small subcutaneous abscesses over the left parietal bone and in the right infraorbital region. Two slightly indurated scars over the forehead, and there is a slightly raised wartlike lesion on the right side of the nose. There are partially healed subcutaneous abscesses on the lips, left ear, and in the right submaxillary region. A large sloughing area is present on the right buttock, which connects with an abscess in the muscles of the thigh. There is also a large abscess over the right knee with extensive undermining of the skin, from which considerable thick reddish pus can be expressed. There are subcutaneous abscesses over both ankles with denudation of bone, and healed and partially healed lesions over both forearms, thighs, legs, and several of the fingers.

Abdominal wall thin, muscles are light brown and dry. There is a small amount of milky fluid in the pelvis. The appendix is free, peritoneum smooth and glistening. Old adhesions between the gall-bladder and colon. The mesenteric glands are large. The diaphragm extends to the fourth right rib and the fourth left interspace. The pericardium contains a large amount of clear fluid. There are bands of fibrous adhesions of the pleuræ.

The thyroid gland is symmetrically enlarged and weighs 65 grams. The cut surface shows an increase in colloid material and fibrous tissue.

In the covering of the thyroid cartilage on the right side there is a small cavity containing purulent material. The cartilage below appears to be normal. The larynx and trachea show no changes.

Over the upper lobe of the right lung are a number of subpleural hemorrhages. The right lung is rather dry, and the tissue filled with small nodules, miliary, or slightly larger in size, with hemorrhagic borders. The lung weighs 545 grams. The left lung shows adhesions at the apex in which are large areas of consolidation. Section shows numerous connected small cavities, 1-1.5 cm. in diameter. The lower lobe answers to the description of the right lung.

The heart is small, and weighs 225 grams. The epicardial fat is replaced by a gelatinous substance and the epicardial vessels are tortuous. The anterior leaflet of the mitral valve shows some thickening along the line of attachment of the chordæ tendineæ. The beginning of the aorta shows a few yellowish plaques. The wall of the left ventricle is 15 mm. in thickness; that of the right ventricle is 4 mm. and flabby.

On removal of the pharynx and esophagus there is opened an abscess cavity posterior to the esophagus; there is erosion of the spinal column from the inferior margin of the fifth dorsal vertebra to the seventh cervical. It is more marked on the left side and extends laterally far enough to involve the anterior surfaces of the second, third, fourth, and fifth ribs. The abscess contains a small amount of yellowish pus.

The lining of the esophagus is smooth. The stomach small and empty. Stomach wall is thicker than normal and rugæ prominent and cannot be obliterated by stretching. The intestines show no changes.

The thoracic duct is patent.

The liver weighs 1,300 grams. It is smooth externally, of normal color. There is a relative increase of intralobular connective tissue, and the centers of the lobules are deep red. The gall bladder contains a considerable amount of bile.

The pancreas weighs 85 grams.

The spleen weighs 300 grams, and is firmly adherent to the diaphragm from which it can be separated only by lacerating the capsule. It cuts easily, is dark red in color, and at several points in the pulp there can be seen small whitish areas, pin-point or less in size which appear to be rather too white and prominent to be Malpighian corpuscles.

The adrenals are normal.

The kidneys weigh 320 grams, smooth externally, capsule slightly adherent, fetal lobulation is unusually well preserved; color and markings are normal. There are several minute cysts in the cortex of the right kidney.

The bladder shows no changes. The prostate is not enlarged, but contains more than the normal amount of fibrous tissue.

The brain externally and on section shows no changes.

HISTOLOGICAL EXAMINATION.

Lungs.—The nodular areas seen at the necropsy resemble miliary tubercles. If one be examined with the high power there is seen in the center a mass of necrotic

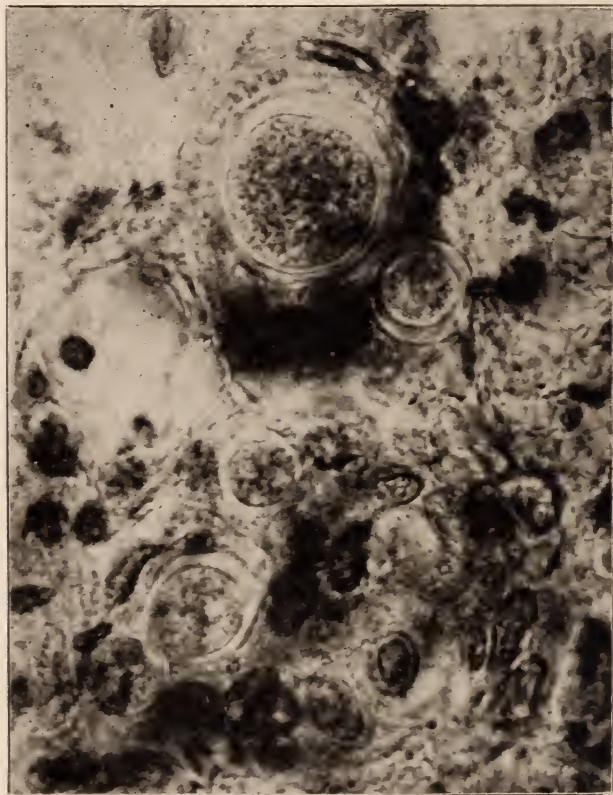


FIG. 4.—Section of lung showing several blastomycetes with characteristic double contours. Hematoxylin and eosin. Photomicrograph, $\times 1,500$.

tissue, containing numbers of polymorphonuclear leucocytes, red corpuscles, desquamated epithelial cells, and many blastomycetes. Immediately surrounding this necrotic focus is a zone of giant cells of the Langerhans type, and embryonic connective tissue cells. As many as 15 giant cells occur in some places and almost without exception they contain blastomycetes in varying numbers. Outside is an area of small round cells with a few connective tissue fibers and distended blood capillaries. Plasma cells are occasionally found. In these areas the alveolar walls are often indistinguishable, and the bronchioles contain large numbers of desquamated epithelial cells, cellular detritus, and in a few instances two or three blastomycetes. Occasionally a bronchiole can be made out at the center of such an inflammatory focus. In the region of one of the minute abscesses a large blood vessel is seen to contain two blastomycetes. Many of the aveoli in the less affected areas contain desquamated epithelium and a cellular detritus; others are markedly emphysematous.

In general these regions of pneumonia are adjacent to the larger blood vessels and bronchioles. By the study of a number of serial sections it is seen that the condition is a true broncho-pneumonia. The inflammatory foci surround the respiratory bronchioles and increase in area toward the periphery of the lung; thus corresponding with the ramifications of the tubules. Furthermore, the alveoli situated farthest from the bronchioles are least affected.

The intima of the larger blood vessels is slightly thickened. The lymph channels contain a considerable amount of black pigment. The visceral pleura is somewhat thickened, and small areas of hemorrhage are seen beneath the endothelial layer.

Retrosophageal abscess.—Sections from the lining of the large abscess show next the abscess cavity, necrotic tissue, and polynuclear leucocytes; surrounding this is an area consisting largely of giant cells containing blastomycetes, together with fibroblasts and numbers of small round cells.

Spleen.—The small whitish, pin-point areas noted in the fresh specimen, prove to be minute collections of blastomycetes surrounded by polynuclear leucocytes and necrotic tissue. No giant cells are seen.

Kidney.—Sections show only a rather marked passive hyperemia. Numbers of sections from various portions of the organs were examined, but no abscesses nor organisms were found.

Thyroid.—Many of the alveoli in the thyroid are enlarged and there is present an excess of colloid material.

Sections from the liver, pancreas, mesenteric gland, adrenal and myocardium show no important changes. The prostrate shows a moderate increase in fibrous tissue with occasional corpora amylacea.

STUDY OF SMEARS AND CULTURES.

Cultures and smears were made of the peritoneal, cerebrospinal, and pericardial fluids, heart's blood, pus from the retrosophageal abscess and from an abscess over the right knee, and of the kidney, spleen, and liver. Coverglass preparations were also made of fluid expressed from affected portions of the left lung. Films made from the heart's blood, pericardial and peritoneal fluids showed no organisms, and cultures remained sterile for four weeks. The *Streptococcus pyogenes* was obtained in coverslip preparations and pure culture from the cerebrospinal fluid, liver, spleen, and kidney, the *Staphylococcus pyogenes aureus* was found in the liver and spleen and in pus from the retrosophageal and knee abscesses. Films prepared from the pus of the two

abscesses just mentioned and from fluid expressed from the pneumonic areas in the lung showed many yeastlike organisms with budding forms, varying in size from 7 to 20 μ in diameter.* Efforts to isolate the yeast from the pus of the abscesses were unsuccessful. The organism was, however, obtained in pure culture from the kidney, although no yeast cells were found in coverslip preparations made from that organ.†

The yeastlike organism obtained postmortem agreed culturally and morphologically with the organism repeatedly isolated from the several abscesses and from the sputum during life. We experienced the same difficulty in obtaining growths from the postmortem material as from the fresh pus. In the first series of cultures growth appeared only after seven days and proceeded slowly, but after the fourth transfer on glucose agar small colonies were visible in three days at 37° C. After growth was once obtained cultures were readily maintained on all the usual laboratory media and showed no tendency to die out.



FIG. 5.—Blastomycetes from a culture in which budding forms are prominent. Glucose agar. Mounted in per cent KOH. $\times 300$.

Morphology.—A typical young culture on glucose agar answers to the following description: There are small white colonies which under a low-power lens are seen to possess numerous aerial hyphæ with usually single terminal and numerous lateral conidia. Coverglass preparations show many doubly contoured segmented threads with homogeneous protoplasm, except that in some of the segments there are from two to four small, spherical, highly refractile bodies about 1 μ in diameter, arranged in the midaxis of the segments. The segments vary from 16 to 29 μ in length and

* A careful examination of material from the lungs was made for tubercle bacilli, but none were found.

† In making cultures from the kidney, as is the routine in this laboratory, a piece of the organ removed under precautions to prevent contamination from either the air or renal pelvis was dropped into sterile broth, and macerated, and this turbid suspension used as material for the inoculation of culture media.

from 3 to 5 μ in width. At various points segments pass off at right angles to the main chain and terminate in a slightly rounded tip, similar to that of the terminal segment of the main stem. At other points in place of lateral segments there are globular structures, from 6 to 8 μ in diameter, connected with the main stem by short, doubly contoured pedicles. These globular offshoots are similar in the relations of their cell walls and protoplasm to the terminal conidia; and both structures are often seen to contain several spherical, highly refractile bodies, 0.5 to 1 μ in diameter, suggesting ascospores in appearance. Besides the hyphal and mycelial portions of the growth there occur in the cultures budding forms similar to those found in the tissues. These budding forms show a characteristic double contour and a more or less granular protoplasm. Occasionally the beginning development of the mycelial threads from one of the budding forms is observed.* (Figs. 5 and 6).



FIG. 6.—Blastomycetes from a culture in which the mycelial forms predominate. The characteristic branching, lateral budding, and protoplasmic granulation can be seen. Glucose agar. Mounted in 1 per cent KOH. $\times 300$.

Cultures.—On *plain* and glycerin agar growths occur in from five to seven days and vary in appearance with the amount of moisture present. In relatively dry cultures the growth is moldlike, elevated, with definite margins, and of a white color from the formation of aërial hyphæ. Surrounding the elevated portion of the growth is a non-elevated rather translucent zone which microscopically is found to consist of numerous interlacing mycelial threads growing in the superficial layers of the medium. Where larger amounts of moisture are present the growth is more pasty, brownish

* Aërial fructification, one of the characteristics of the *oidium hyphomycoides* of Ricketts, was never observed. The proportion of budding forms to hyphæ varied with the kind of media, age of the culture and condition of environment. Lower temperatures, lack of oxygen, marked alkalinity, and the presence of much moisture seemed to favor the formation of mycelium and aërial hyphæ. In cultures in which the reverse conditions obtained budding forms were much more numerous.

in color, and consists almost entirely of mycelial threads. In younger cultures and in those freshly isolated a number of budding forms are found.

On *glucose agar* the growth is more profuse with the formation of mycelium and aerial hyphæ. These hyphæ often form, after three or four weeks, a white, felt-like mass resembling a plug of cotton. Here, as in other media, the mycelia tend to grow down into the deeper layers of the medium which gradually becomes rich golden brown in color.

In *broth* (1 per cent acid to phenolphthalein) there forms a fluffy, coherent, globular growth consisting of many very long mycelia radiating out in all directions from the central point of inoculation, the supernatant liquid remaining clear.

Gelatin is not liquefied after five weeks.

Litmus milk after 10 days is slightly alkaline and proteolysis without coagulation has occurred.

On *potato* the growth is rather more rapid than on other media, and there are many aerial hyphæ. In certain of these cultures the hyphæ persist, but in others for some reason they begin after a few days to disappear. The growth becomes brownish-white in color with a more or less wrinkled, elevated surface; and microscopically the mass is found to consist in large part of the budding forms with but few mycelial threads or hyphæ.*

Freshly isolated cultures grow slightly better on *Loeffler's blood serum* than on agar media, but after the first transfer, blood serum gives no better growths than the other media. Cultures of the organism in fermentation tubes containing sugar-free broth to which 1 per cent of dextrose, levulose, saccharose, lactose, galactose, mannite, and nutrose have been added show no gas formation in any of the tubes during four weeks of observation. Freshly isolated cultures grow somewhat more readily at 37° C. than at 20° C. with a tendency to the budding form. At 20° C. the older cultures grow as rapidly as at 37° C., and the growths consist almost entirely of mycelium and aerial hyphæ. The optimum reaction of the culture media is from 0.5 per cent to 1.0 per cent acid to phenolphthalein. In agar made 0.5 per cent alkaline growth is less abundant, becoming scant at 1.5 per cent alkali and not occurring at all when higher degrees of alkalinity are employed.

ANIMAL EXPERIMENTS.

A number of guinea-pigs and rabbits were inoculated with pure cultures of the blastomyces obtained from the abscesses during life with varying results. Subcutaneous inoculations were usually followed by local abscess formation. Such abscesses as a rule contained yellowish cheesy material in which the blastomycetes were present in considerable numbers in the budding form. Even when cultures consisting chiefly of mycelium were injected, examination of the abscess after a few days showed only budding forms. In one or two cases subcutaneous injections failed to produce a typical abscess, only a slight induration remaining at the site of puncture. Likewise intraperitoneal injections of cultures were often negative no lesion whatever, being discoverable in three or four weeks after inoculation. In one rabbit, however, general miliary blastomycosis followed the intraperitoneal injection of 3 c.c. of a broth suspension of blastomyces. The organisms were demonstrated in the liver

* The same phenomenon was observed in the case of one or two agar cultures, but we are unable to define the exact conditions under which the change took place.

lungs, kidney, and spleen. The intravenous injection of a suspension of the blastomyces, using the marginal ear vein, resulted in two cases in rather extensive lesions in the lungs in which the blastomycetes could be demonstrated in sections. Many of the organisms were apparently undergoing degeneration and disintegration, having lost their double contour and protoplasmic granulation. Extensive hemorrhages were present in the lung tissue, and marked passive congestion in the other organs. The process seemed to be largely due to mechanical obstruction in the vessels of the lungs.

In all the animal lesions, the absence of giant cells was a noticeable feature.

SUMMARY.

Etiology.—We were able to isolate from the several lesions in this case a characteristic yeastlike budding organism which morphologically and culturally belongs in the general class *Oidium* as defined by Ricketts, and of the several members of this group, resembles most closely the form isolated by Gilchrist and Stokes,¹ and described by them under the name *Blastomyces dermatitidis*.

The organism was repeatedly demonstrated and obtained in pure culture from the abscesses at different points and from the sputum during life. Postmortem the same organism was obtained in culture from the kidney and demonstrated in sections from the lungs, spleen, and walls of the abscess cavities, and also in pus from the abscesses, and bronchial secretion. It appeared only in the blastomycetoid form in the tissue, no sporulation nor mycelium formation being observed. When once obtained in culture it grew readily on artificial media, in both the mycelial and budding forms.

The disease was reproduced in animals by the inoculation of pure cultures of the blastomyces, and the organism again recovered postmortem from the lesions.

Finally we feel that the tubercle bacillus was definitely excluded as a causal factor in the disease. There was no family or personal history of tuberculosis. Many examinations of the sputum, urine, and pus from the abscesses by staining methods and by inoculation into guinea-pigs were made for tubercle bacilli with uniformly negative results. Examinations of smears and sections from the various organs postmortem likewise gave negative results as to tubercle bacilli.

The immediate cause of death was a terminal mixed streptococcus and staphylococcus septicemia which probably hastened death by a few days only.

¹ *Jour. Exper. Med.*, 1898, 3, 1, p. 53.

Clinical features.—The disease showed the characteristics of a chronic infection with gradual onset and a degree of general intoxication relatively slight when the extent of the lesions is taken into account. Diagnoses of both syphilis and tuberculosis had been made, but all who had seen the patient agreed that the case was not typical of either. On admission to the hospital, the history atypical of tuberculosis or syphilis, the number, size, and indolent character of the abscesses, and the bloody appearance of the pus suggested the probable diagnosis of generalized blastomycosis, which was at once confirmed by finding large numbers of the blastomycetes in the pus.

The infection atrium seems in all probability to have been the respiratory tract. The patient was an intelligent man, who, though engaged in manual labor, had always been unusually cleanly in his habits. He was certain that he had had no cutaneous lesions whatever previous to the appearance of the first abscess in the groin. This statement was confirmed by his wife. From his description of the succeeding lesions it seems certain that they were of the same nature as the first, and that they were due to successive localizations of the infection agent derived from some common source, probably a lesion in the lungs. It is true that during the first six months of the disease, no pulmonary symptoms were observed, but a lesion may still have been present without producing local signs, in the same way that a tuberculous focus may remain unrecognized for long periods. On admission the lungs were examined with care in view of the probability of primary pulmonary involvement, but no lesion was made out at this time. A few weeks later, however, definite pulmonary symptoms appeared together with physical signs and many blastomycetes in the sputum. It is noteworthy that the extensive pulmonary involvement found at autopsy was out of proportion to the physical findings during life.

It seems certain that dissemination of the organisms took place in very large measure, if not entirely, by the blood. The successive localizations of the blastomycetic abscesses at one point and then at another, distant from the first, the situation of the lesions chiefly on the extremities, the occurrence of miliary abscesses in the spleen, the finding of blastomycetes in sections within the blood vessels of

the lung, and the absence of regional glandular involvement all speak for a condition of blastomycemia. Carefully conducted blood cultures on two occasions failed to demonstrate blastomycetes, but this is not to be wondered at when we consider the relatively large size and small number of the organisms in comparison with cases of pyemia due to bacterial infection. It is altogether likely that the organisms were carried to practically all regions of the body, but only at certain points was the local condition of the tissue such as to allow their multiplication with the formation of abscesses.

The course was progressively downward with slowly increasing anemia and emaciation. There was a constant moderate leucocytosis even when no secondary infection could be demonstrated. A slight absolute eosinophilia was present. This was observed by one of us in another case of generalized blastomycosis.

The lesions were as a rule painless except at the very beginning, when there was some tenderness over the indurated nodules. In the lesions in which the primary focus was subperiosteal the patient complained of a considerable amount of pain. In several instances the development of the small subcutaneous nodules through the successive stages of growth, softening, fluctuation, enlargement, and then rupture of the abscess, with formation of a sinus and finally a slowly healing ulcer, was observed. Where the abscesses were deep seated there was occasionally considerable dissection along the intermuscular fasciæ. Thus an abscess which formed beneath the calf muscles discharged just above the ankle, and a second abscess originating beneath the gluteal muscles pointed in the popliteal space.

Of the two superficial ulcers on the leg, it may be stated that though they showed one or two miliary abscesses at the periphery these did not contain the organisms, and the ulcers themselves were not typical of blastomycetic dermatitis. They healed uniformly from the periphery inward and apparently had resulted, as the patient stated, from the breaking-down of the skin over subcutaneous abscesses. The lesions in this case were thus practically all subcutaneous or still more deeply seated.

Anatomy.—Whenever examined, the pus from the abscesses, and later the sputum, contained the blastomycetes in considerable

numbers. In some of the reported cases, it has been difficult at times to demonstrate the organisms in pus from the lesions. This was true in an unreported case seen by one of us in which there was a large subscapular abscess and a second abscess in the lumbosacral region, both containing large amounts of pus in which organisms were demonstrated only after long search. Cultures also were sterile on the first trial, and the patient left the hospital before more material could be obtained.

In the histology of the lesions a marked feature was the presence of large numbers of giant cells. This was noted particularly in the lung lesions, in which the changes were very similar to those found in acute pulmonary tuberculosis. The absence of tubercle bacilli was determined by careful search. In the lesions produced in animals, on the other hand, giant cells were not noted.

In addition to the general miliary blastomycosis of the lungs, there were several areas of ulceration with cavity formation. The present is the first case in which this has been noted. In general the distribution of the inflammatory areas in the lungs corresponded to the course of the smaller bronchi and bronchioles, and the process seemed to be true broncho-pneumonia. This fact is in accord with the view that the pulmonary infection was an inhalation process.

The superficial erosion of the vertebræ apparently followed a retroesophageal abscess and did not begin primarily in the bone.

The absence of amyloid changes in the various organs deserves mention, in view of the extensive and long-continued suppuration. This is in marked contrast to the findings in the otherwise rather similar case reported by Bassoe,¹ in which in almost all the organs amyloid changes were advanced.

¹ *Loc. cit.*

THE RÔLE OF PHAGOCYTOSIS IN THE PNEUMOCOCCIDAL ACTION OF PNEUMONIC BLOOD.*†

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SYNOPSIS.

INTRODUCTION.

PHAGOCYTOSIS OF PNEUMOCOCCI.

Technique.

The Effect of Normal Serum.

Variation in Susceptibility of Pneumococci to Phagocytosis.

EFFECT OF HEAT UPON PHAGOCYTOSIS.

Heating the Serum.

Heating the Phagocytes.

Heating the Pneumococci.

THE OPSONIC POWER OF PNEUMONIC SERUM.

COMPARATIVE PHAGOCYTIC VALUE OF NORMAL AND PNEUMONIC LEUCOCYTES.

RELATION OF VIRULENCE AND RESISTANCE TO PHAGOCYTOSIS.

THE PNEUMOCOCCIDAL ACTION OF LEUCOCYTIC BLOOD.

CONCLUSIONS.

INTRODUCTION.

OUR knowledge of the mechanism of invasion and healing in pneumococcus infections generally, and more particularly in lobar pneumonia, is as yet very incomplete. To penetrate more deeply into such mechanism and to apply newer developments in immunology, the result primarily of the researches of Wright and Douglas,¹ is the object of this investigation. This paper deals especially with phagocytosis of pneumococci *in vitro* and with the part played by phagocytosis in the pneumococcidal action of human blood, especially pneumonic, following closely the lines pursued by Ruediger² in his study of the streptococcidal effect of human blood, and by Hekt en³ of the anthracidal action of dog blood.

PHAGOCYTOSIS OF PNEUMOCOCCI.

Technique.—In the phagocytosis experiments, unless otherwise specified, the technique, in the main, is that which was first worked

* Received for publication June 2, 1906.

† This work was aided, thanks to Dr. F. Billings, by a grant from the Medical Commission for the Investigation of Acute Respiratory Diseases of the Department of Health of the City of New York.

¹ *Proc. Roy. Soc.*, 1903, 72, p. 357, and 1904, 73, p. 128.

² *Jour. Infect. Dis.*, 1906, 3, p. 156.

³ *Ibid.*, p. 102.

out by Leishman¹ and used so extensively later by Wright and Douglas,² by Hektoen and Ruediger³ and others.

Leucocytes in defibrinated or washed blood suspended in serum are used as phagocytes. The pneumococci are added either in suspensions of 0.85 per cent NaCl solution or in 24-hour broth cultures. Definite quantities of defibrinated blood or washed leucocytes, serum, and suspension of bacteria are mixed by means of graduated pipettes and then incubated at 37° C. The smears may be made at different intervals. They should be made rather thin and dried quickly, so as to spread the leucocytes over a large surface, thus making it possible to obtain accurate counts when a relatively large number of organisms are taken up. This is most conveniently done by placing on the slide a large platinum loopful of the suspension and then spreading it with tissue paper cut in suitable squares. The smears are stained either by Leishman's or Wright's modification of Romanowsky's method. Both have been found equally satisfactory, although it is more difficult to prepare the former. The numbers in the tables represent the average number of pneumococci contained in each leucocyte, obtained by counting the number of bacteria in from 20 to 50 leucocytes.

Previous investigators in this field have commonly completely disregarded the influence upon phagocytosis that movement of the mixtures might have. Red blood corpuscles and leucocytes soon settle to the bottom of the tubes when kept quiet and hence the distribution of leucocytes, serum, and organisms in the mixtures necessarily becomes unequal. In order to obviate this and to approximate more closely *in vitro* the conditions present in the circulation, I devised a small shaking machine that could be placed in the incubator. With this modification in the technique phagocytosis is perceptibly increased and the results obtained would seem more reliable, because phagocytes and pneumococci are brought into closer contact. The shaking must not be too vigorous, otherwise the leucocytes are broken up. About 120 to 150 vibrations per minute with a stroke of 3 mm. is the most satisfactory. This is just sufficient to keep the corpuscles and leucocytes equally suspended and does not break up the leucocytes.

¹ *Brit. Med. Jour.*, 1902, 1, p. 73.

² *Loc. cit.*

³ *Jour. Infect. Dis.*, 1905, 2, p. 128.

The effect of normal serum.—The quantitative effect of normal human serum on phagocytosis of pneumococci is shown in Table 1. As the amount of serum is diminished the number of pneumococci ingested falls until when no serum is added there is no phagocytosis.

TABLE 1.
QUANTITATIVE EFFECT OF SERUM ON PHAGOCYTOSIS.

Washed normal blood		0.2 c.c.		Pneumococcal suspension		0.2 c.c.		Phagocytosis (after 1 Hour)	
Normal serum	0.2	c.c.	+ NaCl sol.	0.0	c.c.
"	"	0.1	" + "	"	0.1	"	.	.	40
"	"	0.05	" + "	"	0.15	"	.	.	31
"	"	0.025	" + "	"	0.175	"	.	.	27
"	"	0.0125	" + "	"	0.1875	"	.	.	10
"	"	0.0	" + "	"	0.2	"	.	.	3
"	"	0.0	" + "	"	0.2	"	.	.	0

Variation in the susceptibility of pneumococci to phagocytosis.—The results of my early experiments quickly showed that there is great variation in the susceptibility to phagocytosis, *in vitro*, of various strains of pneumococci (Table 2).

TABLE 2.
PHAGOCYTOSIS OF PNEUMOCOCCI BY HUMAN LEUCOCYTES.

DEFIBRINATED BLOOD + PNEUMOCOCCAL SUSPENSION		NUMBER OF PNEUMOCOCCI TAKEN UP PER LEUCOCYTE IN				
		15 Min.	1 Hr.	2 Hrs.	3 Hrs.	8 Hrs.
Pneumococcus	O	30	23	31	48	85
"	Cl	14	20	24	25	31
"	N	0.4	0.9	3.2	6	12
"	S*	0	0	0	0	0.8

*This highly virulent pneumococcus was suspended in serum for three and one-half hours previous to the experiment, yet it failed to become sensitized.

Strains which have been kept upon artificial media and have lost their virulence are taken up freely (Pneumococcus O and Cl, Table 2). Others possessing a high degree of virulence for rabbits resist being taken up after 8 to 12 hours' contact with the serum (Pneumococcus S). Forty strains of pneumococci have been tested. They were obtained chiefly from the blood and sputum of pneumonia patients. All but four resisted phagocytosis at first, but usually became susceptible soon after cultivation in artificial media. In the degree they became susceptible to phagocytosis they, without exception, lose virulence.

At first smears were made at frequent intervals in order to ascertain the exact sequence of events, especially with reference to the

speed with which leucocytes take up various pneumococci, whether there might be a demonstrably rapid intraphagocytic lysis of pneumococci and whether at first nonsusceptible pneumococci become so after a longer contact with opsonin. We see that in the case of *Pneumococcus N* (Table 2) there is practically no phagocytosis until at the end of three hours, when there is an average of six in each leucocyte; while at the end of eight hours there are 12. Is this difference between the number taken up at the end of three and of eight hours the result of an active continuous phagocytosis or the result, perhaps, of an intraphagocytic multiplication of pneumococci, or of both combined? To throw some light upon this point washed leucocytes mixed with serum and pneumococcal suspensions have been studied microscopically on the warm stage. Not only have active amœboid movements been observed so long as 12 hours, but actual ingestion of pneumococci has been seen, thus showing that the increase is, at least in part, the result of phagocytosis and not the sole result of an intraphagocytic multiplication alone. That growth may occur within the leucocytes at times can scarcely be questioned, however. In mixtures in which the organisms are taken up freely, the leucocytes first fill themselves, then burst, as it were, and finally seem to become the center of a vast colony. The question of intraphagocytic destruction of pneumococci is discussed in the later part of the paper.

EFFECT OF HEAT UPON PHAGOCYTOSIS.

Heating the serum.—Opsonin for pneumococci is considerably diminished by heating undiluted serum to 56° C. for 30 minutes, and when serum is heated to 60° for one and one-half hours there is a very marked diminution in this substance. Normal and pneumonic serum behave alike in this respect. It would thus seem that the opsonins in these sera are probably identical, and quite resistant to heat. It must be remembered, however, that the character of the organism with which one works may modify the results materially. Thus a particular pneumococcus strain which in the beginning was not at all susceptible to phagocytosis in the presence of serum, finally by long cultivation upon artificial media (seven months) became so freely taken up that carefully washed leucocytes suspended in normal salt solution took them up readily.

TABLE 3.
EFFECT OF HEATING LEUCOCYTES UPON PHAGOCYTOSIS.

Washed Blood Normal Serum Pneumococcal Suspension	} Equal Parts		Phagocytosis in One Hour
Washed blood unheated	.	.	17
" " heated at 40° C. for 15 min.	.	.	18
" " " 45 " 15 "	.	.	8
" " " 50 " 15 "	.	.	3*
" " " 55 " 5 "	.	.	1.2*
" " " 60 " 5 "	.	.	0*
" " " 60 " 30 "	.	.	0

* Leucocytes surrounded by pneumococci.

Heating the pneumococci.—Heating the pneumococci has practically no effect upon their susceptibility to phagocytosis, which is in accord with the findings of Reque¹ in regard to heated diphtheria bacilli. Pneumococci of high virulence and insusceptible to phagocytosis are not made susceptible by subjecting them to 90° C. for 30 minutes.

EFFECT OF HEATING PNEUMOCOCCI ON PHAGOCYTOSIS.

Non-virulent pneumococcus, unheated	.	.	40	heated to 90° C. 30 m.	.	.	37
Slightly virulent " " " 10 "	.	.	10	" " 90 30 "	.	.	7
Highly " " " 0 "	.	.	0	" " 90 30 "	.	.	0

Heating the leucocytes.—When the leucocytes are heated they soon lose their phagocytic power (Table 3). The effect upon the leucocyte is the same whether one heats it in washed blood, suspended in normal salt solution, or in serum. In carrying on these experiments it was noted that when washed pneumonic leucocytes were heated side by side with washed normal leucocytes, the former were more resistant. This seems especially true of leucocytes which are obtained from cases of infection which terminate favorably.

Pneumonic leucocytes when heated at 60° C. for five minutes no longer possess any phagocytic power. Normal leucocytes usually cease to take up pneumococci at a temperature from three to five degrees lower. By the aid of the shaking machine, which serves to bring leucocytes and pneumococci in closer contact, the following interesting observation was made. When pneumonic leucocytes are heated from 50° to 60° C. for from 5 to 15 minutes and suspended in pneumonic serum, they no longer take up pneumococci, but appear markedly positively chemiotactic so that they become surrounded often by large numbers of pneumococci (see Plate 22). This frequently gives the impression of an agglutination, and suggests to the

¹ Jour. Infect. Dis., 1906, 3, p. 414.

observer that the leucocyte may be the source of the agglutinin. Polymorphonuclear leucocytes only show this tendency. Normal leucocytes, heated in normal serum also do this, but to a lesser degree and at a temperature from 3° to 5° C. lower. To bring out this phenomenon well, and especially to bring out the difference in behavior of the pneumonic and normal leucocytes, the use of a shaking machine (vibrations should be about 120 per minute, with a stroke of 3 mm.) is necessary. A pneumococcus which is freely susceptible to phagocytosis and the presence of an active serum is essential to bring out this phenomenon. Leucocytes suspended in heated serum or in normal salt solution do not show this perileucocytic arrangement. Hence opsonin or some analogous substance is necessary.

That the rather remarkable difference in the behavior of the pneumonic and normal leucocytes is due to the leucocytes themselves is shown by the facts that heated normal leucocytes in pneumonic serum behave exactly the same way as in normal serum, and that pneumonic leucocytes placed in normal serum behave exactly as they do in pneumonic serum. The leucocytes from two cases of appendicitis and one case of puerperal sepsis behaved in this respect like the leucocytes of pneumonia.

OPSONIC POWER OF PNEUMONIC SERUM.

Having shown that phagocytosis of pneumococci *in vitro* is proportional to the amount of serum present, and that the opsonin in normal and pneumonic sera is probably identical, the question whether there is an actual increase of pneumococco-opsonin in pneumonia was carefully studied. Table 4 gives the results of some of the experiments on this point. It shows no appreciable increase in the opsonic powers of pneumonic serum during any part of the disease, but does show a diminution in fatal cases. Since organisms freely susceptible to phagocytosis were chiefly used with undiluted serum, these results might be explained upon the score that the number of organisms opsonized in both cases is so large that differences might not be detected. On this account strains of pneumococci of a grade of virulence such that they were taken up only in small numbers under the conditions of the experiment and dilutions of serum were used. In this way the opsonin content of the serum

TABLE 4.
THE OPSONIC POWER OF PNEUMONIC SERUM WITH RESPECT TO PNEUMOCOCCUS.

WASHED NORMAL BLOOD	2	VOL.
SERUM	2	"
PNEUMOCOCCAL SUSPENSION	1	"

DAY OF DISEASE	PHAGOCYTOSIS	
	Pneumonic Serum	Normal Serum
Day after crisis	38	41
Atypical crisis	22	20
Day after crisis	45	40
During crisis	33	43
Fifth day	32	43
Twelfth day	56	53
" " *	57	56
Sixth day	28	32
Day after crisis	23	20
Third day	28	26
Day of death, seventh	11	15
" " "	10	12
" " "	20	24
Day after crisis	45	40
" " "	12	10
Second day after crisis	9	6

* Homologous organism.

of three cases of lobar pneumonia was determined upon three different occasions with the result that a perceptible increase, but not a large one, over that of the normal was obtained upon the day after crisis in two of the cases. The other failed to show increase at any period. A further study of this point was undertaken by my colleague, Dr. Wolf, whose results seem to indicate that very early in pneumonia the opsonin is diminished, then it gradually increases somewhat until it reaches its maximum, usually upon the first day following crisis. At this time there is apparently a perceptible increase. Whether this increased phagocytosis in pneumonic serum is the result of an actual increase of opsonin or due to an agglutination is difficult to say, especially since pneumococcus agglutinins in pneumonia seem to be most abundant at this time, as shown in my previous paper.¹ The diminution observed very early in the disease and in the fatal cases, however, can scarcely be attributed to anything else than an actual diminution of opsonin.

COMPARATIVE PHAGOCYTIC VALUE OF NORMAL AND PNEUMONIC LEUCOCYTES.

In a long series of experiments, to be referred to later in this paper, in which defibrinated blood, normal and pneumonic, were studied side by side with reference to their pneumococcidal effect and

¹ *Jour. Infect. Dis.*, 1904, 1, p. 280.

their relative phagocytic power, it was noted that pneumonic blood containing from two to even four times as many leucocytes per cubic millimeter as the normal would give as high and even a higher degree of phagocytosis.

At first this was thought to be due to increase in opsonin in the pneumonic serum, but careful study rather negated this idea (see Table 4), and it seemed imperative that the leucocytes be taken into account. In the literature I am unable to find detailed observations concerning physiological differences between leucocytes in infections and normal leucocytes. Metchnikoff, while he considers leucocytes in immune animals more actively phagocytic, attributes this to "stimulins" in the serum. Most investigators consider the leucocytes of infections on a par with normal leucocytes.

Care was exercised to control all possible sources of error. The samples of normal and pneumonic bloods were obtained as nearly as possible at the same time, and subjected to the same conditions of temperature. As it was essential to use washed leucocytes, the bloods were washed exactly in the same way by using large quantities of 0.85 per cent NaCl solution, mixing thoroughly each time. The washing was repeated four times. The number of leucocytes in the normal blood must be the same as those in the pneumonic blood in order that the results may be directly comparable. This was done by carefully pipetting off the proper proportion of the top layer of the centrifugated blood, rich in leucocytes, and adding the proper amount of the deep layer, containing few leucocytes, until the leucocyte counts showed an equal number in both. Table 5 shows that phagocytosis by pneumonic leucocytes is perceptibly greater than by normal leucocytes subjected to the same conditions. Strains of pneumococci which are freely taken up by both are taken up in larger numbers by the former; other strains which are more virulent and taken up to a slight extent are also taken up more freely by pneumonic leucocytes; while still other strains which are not taken up at all by normal leucocytes are taken up to some extent by pneumonic leucocytes. It is very difficult to obtain organisms of just the proper virulence to bring out this difference; numerous tests failed, but by repeated efforts the happy combination was obtained. The degree of phagocytosis in Table 5 is high in most instances, yet by making

TABLE 5.
COMPARATIVE PHAGOCYTIC VALUE OF NORMAL AND PNEUMONIC LEUCOCYTES.

[illegible]

thin smears and drying them quickly, spreading out the leucocytes widely, and using a Zeiss lens the counts obtained are sufficiently accurate to be reliable. To satisfy every demand it should be stated that the same comparative results were obtained by using falling quantities of serum.

That this increased phagocytic power of pneumonic leucocytes is not due to an increase in the opsonic power of pneumonic serum alone, is certain because normal serum answers practically as well. Whether it is due to an increased motility or to an increased sensitiveness is difficult to say. In the warm stage no perceptible increased motility could be detected. This is, however, a crude test and only great differences could be made out in this way. That the higher phagocytic power of pneumonic leucocytes is due rather to the condition of the leucocytes themselves, than to increased sensitiveness to opsonic influences, is indicated by the result of the following experiments. A pneumococcus, which has been cultivated upon artificial media for over seven months, is so freely susceptible to phagocytosis that carefully washed leucocytes, suspended in normal salt solution may take up the cocci. Here, in the absence of serum, the greater phagocytic activity of the pneumonic leucocytes was also evident, and this difference cannot be explained upon the score of an increased sensitiveness to opsonic influences. Is this increased phagocytic activity of pneumonic leucocytes specific for the pneumococcus, or is it a general manifestation? It is probably a general manifestation, for

the reason that leucocytes obtained from other infections, e. g., puerperal sepsis and appendicitis show practically the same increase in phagocytic power as those from the pneumonia, and is perhaps due to the preponderance of younger, and hence more active, forms.

To summarize briefly, then, I have shown: (1) That pneumonic leucocytes are more resistant to heat than normal leucocytes. (2) When heated just sufficiently to lose their phagocytic power they are more positively chemiotactic. (3) They not only take up pneumococci which are freely susceptible to phagocytosis in larger numbers, but take up strains that resist phagocytosis by normal leucocytes. (4) They are more actively phagocytic for certain rare strains of pneumococci in the absence of serum. Whether this increased activity of the leucocyte in pneumonia is more marked as the disease progresses in the favorable cases and less so in the fatal cases might be an interesting field for further study. Judging, however, from the limited number of observations I have made upon this point it seems that no such difference is demonstrable. The leucocytes in several cases that died seemed as active as those obtained before, during, or after crisis.

RELATION OF VIRULENCE AND RESISTANCE OF PNEUMOCOCCI TO PHAGOCYTOSIS.

As stated, there is a close relation between virulence and resistance to phagocytosis of pneumococci.

Cultivation of virulent pneumococci upon artificial media is generally followed by reduction of virulence and increasing susceptibility to phagocytosis. Attempts were made to restore lost virulence and thus see whether resistance to phagocytosis also returned. At first repeated efforts failed. However, by making blood cultures from the heart's blood 12 hours after inoculation of rabbits and promptly reinoculating the isolated organism, I have been able to restore the lost virulence of a pneumococcus sufficiently so that 5 c.c. of a 24-hour broth culture proved fatal in 48 hours to a rabbit weighing 1,600 gms. In the proportion that virulence was restored, resistance to phagocytosis returned. A chronological summary of one of these experiments is given in Table 6.

It would seem that the property of virulent pneumococci which prevents phagocytosis may be restored by animal inoculations after

TABLE 6.
RELATION OF VIRULENCE AND RESISTANCE TO PHAGOCYTOSIS OF PNEUMOCOCCUS E.

Date, etc.	Phagocytosis	Rabbit Virulence of 24-Hour Broth Cultures
Oct. 30. original culture.....	0	1 c.c. fatal in 12 hours
Nov. 2. organism cultivated in plain broth.....	7	5 c.c. " " 24 "
" 13. " " agar.....	18	
" 27. same as above.....	33	10 c.c. not fatal
Dec. 14. organism passed through seven rabbits.....	3 7	5 c.c. fatal in 48 hours
Jan. 18. organism cultivated upon blood agar. Trans- ferred only once.....	31	10 c.c. not fatal
Apr. 22. organism kept on blood agar without transfer for 3 mos.	10.5	

it has been lost in artificial cultivation. It may be pointed out, however, that the result obtained might be due, at least in part, to a sifting-out of the non-virulent organisms which perish in the animal, while the few which survive, presumably because of greater virulence, become more and more numerous.

I have not been able to establish any definite relation between the presence of the capsule of pneumococci and virulence. It also seems that the capsule has no special influence upon the susceptibility to phagocytosis, because capsules have been demonstrated in strains which are taken up freely, and on the other hand, no capsules may be demonstrable in the case of cocci that resist phagocytosis.

In the course of the work a number of efforts were made to determine whether pneumococci isolated from the blood and not susceptible to phagocytosis in normal and heterologous pneumonic blood might not be taken up when acted on by the homologous serum. In no instance has this been demonstrable.

Just as blood-containing media conserve the virulence of pneumococci, so also they prolong the period until the recently isolated organisms become susceptible to phagocytosis. Thus, four days' growth in plain broth was sufficient to render an organism susceptible, while another organism (obtained from Dr. Wolf) was still insusceptible after daily transfers upon blood agar slants for over five months. Until recently it also possessed a rather high degree of virulence. Suspending this and the homologous organism in pneumonic serum for 24 hours rendered the former freely, and the latter moderately, susceptible to phagocytosis. The question of the absorption of pneumococco-opsonin by virulent and non-virulent pneumococci will be considered in a separate paper.

THE PNEUMOCOCCIDAL EFFECT OF NORMAL AND PNEUMONIC
SERUM AND BLOOD.

In attempting to explain crisis in pneumonia a minute study of the effect upon virulent and non-virulent pneumococci of (1) the defibrinated pneumonic blood; (2) the serum, heated and unheated; (3) the washed corpuscles in normal salt solution or recombined with serum, was made, the results being controlled by analogous experiments with normal blood. A year ago I noted that while the normal and pneumonic serum and normal blood had no pneumococcidal effect, the defibrinated blood of pneumonia patients during crisis had marked effect, 0.5 c.c. destroying about 50 millions of pneumococci in four hours. Heating the defibrinated blood to 50° C. for 30 minutes destroyed the pneumococcidal effect. These results were especially interesting because the blood shortly before and soon after crisis appeared to have no such action, and it seemed as if there was present in the blood during crisis something which destroyed pneumococci. The result of one of these experiments is given in Table 7. This assumption had to be modified, however,

TABLE 7.
PNEUMOCOCCIDAL EFFECT OF DEFIBRINATED PNEUMONIC BLOOD DURING CRISIS.

DEFIBRINATED PNEUMONIC BLOOD 0.5 C.C. + PNEUMOCOCCI	COLONIES IN BLOOD AGAR PLATES				
	At Once	1 Hr.	3 Hrs.	10 Hrs.	24 Hrs.
During crisis.....	5,000	600	0	10	0
" " (heated, 50° C. 30 min.).....	5,200	5,350	7,000	8,000	8,100
12 hrs. before crisis.....	2,750	2,500	4,500	5,500	8,500
2 days after crisis.....	1,500	1,250	2,500	3,500	4,500
Pneumonic serum during crisis.....	2,100	2,950	2,200	4,500	6,000

when the same results were obtained later with leucocytotic pneumonic blood obtained before as well as after crisis. In attempting to determine the mechanism by which pneumococci are destroyed by defibrinated blood a closer study of the effects of normal and pneumonic serum heated and unheated was necessary. I have shown that normal and pneumonic serum have apparently no pneumococcidal power. When pneumococci of low vitality are used, rather large numbers must be inoculated, otherwise growth may fail.

Table 8 shows the growth of pneumococci in normal serum. Heating the serum to 56° C. for 30 minutes has not the slightest effect

TABLE 8.
EFFECT OF NORMAL SERUM UPON PNEUMOCOCCI.

NORMAL SERUM 0.5 C.C.	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hr.	2 Hrs.	12 Hrs.	24 Hrs.
R + pneumococcus R 51a.....	4,000	4,500	4,000	5,600	4,500
R (heated) + pneumococcus R51a.....	4,200	4,000	4,000	5,400	4,600
R + pneumococcus E ²	2,000	1,700	3,580	∞	∞
R (heated) + pneumococcus E ²	2,600	2,450	5,000	∞	∞
H + pneumococcus Otto.....	300	50	1,500	5,400	∞
H (heated) + pneumococcus Otto.....	500	300	400	8,600	∞
R + pneumococcus E ²	1,850	1,800	2,000	6,000	300
R + hemoglobin + pneumococcus E ²	2,000	2,400	5,000	∞	∞
Control broth + pneumococcus E ²	3,800	3,000	3,500	∞	∞

upon it as a culture medium for pneumococci. The primary drop is small, and when a little hemoglobin is added it does not seem to occur.

TABLE 9.
EFFECT OF PNEUMONIC SERUM UPON PNEUMOCOCCI.

PNEUMONIC SERUM	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hrs.	3 Hrs.	12 Hrs.	24 Hrs.
3rd day + pneumococcus N.....	1,200	1,050	1,400	1,560	8,000
" (heated*) + pneumococcus N.....	1,250	1,200	1,370	1,700	8,500
4th day + pneumococcus N.....	1,050	1,300	1,200	1,700	3,000
" (heated*) + pneumococcus N.....	1,100	1,400	1,350	1,500	2,400
During crisis + pneumococcus E ²	2,100	1,450	3,500	6,700	∞
" (heated†) + pneumococcus E ²	3,240	6,700	8,000	∞	∞
" " + pneumococcus O.....	200	110	100	2,500	4,600
" " (heated†) + pneumococcus O.....	100	95	90	1,800	3,700
Day after crisis + pneumococcus R51a.....	3,500	3,000	5,000	∞	∞
" (heated†) + pneumococcus R51a.....	2,500	2,255	3,000	∞	8,000
4 days after crisis + pneumococcus E ²	3,240	7,000	8,000	∞	∞
10 days after crisis (heated†) + pneumococcus N.....	1,650	1,400	1,500	1,700	1,850
6th day + pneumococcus E ²	1,700	1,600	2,300	300	15
" " + hemoglobin + pneumococcus E ²	1,900	2,400	4,000	∞	∞

* 60° for one and one-half hours.

† 56° for 30 minutes.

Table 9 shows that the clear pneumonic serum, heated or unheated, is not pneumococcidal, no matter at what period of the disease it is obtained. The primary drop is somewhat more marked than in the normal serum, especially as crisis is reached and soon thereafter. This may be due to agglutination of pneumococci. Heating the serum has no effect. The addition of hemoglobin or red corpuscles materially aids the growth of the pneumococcus here also. In order to exclude all possibility of complement-amboceptor action a series of tubes, each containing 0.5 c.c. serum, were inoculated with 25 to 200 pneumococci, the whole quantity being plated in each instance.

There was usually a slight fall, then a rise in the number of organisms, just as in the experiments above. Hence the technique employed as represented in the tables is considered reliable.

Further, it was thought possible that the serum during an attack of pneumonia which terminates favorably might acquire pneumococcal properties for the strain of pneumococci which is responsible for the infection, but have no such power for other strains. It was found, however, that pneumococci grew in the homologous sera exactly like those from other sources. In two cases the organisms were isolated from the blood the day previous to crisis and tested with the serum during crisis. In the third the organism was isolated the day after crisis and tested with the homologous serum the day following. The organisms used in these experiments were of all degrees of virulence.

The serum alone not being pneumococcal the explanation of the destructive effect of pneumonic defibrinated blood must depend on other factors than the serum alone.

TABLE 10.
EFFECT OF NORMAL DEFIBRINATED BLOOD UPON PNEUMOCOCCI.

NORMAL DEFIBRINATED BLOOD 0.5 C.C.	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hrs.	3 Hrs.	10 Hrs.	24 Hrs.
B + pneumococcus R 51 ^a	1,540	1,250	150	7	0
B (heated *) + pneumococcus R 51 ^a	3,000	3,500	4,500	6,000	∞
R " + " " " "	3,200	1,250	1,600	2,000	2,500
R " + " " " "	4,000	4,500	3,200	3,500	4,000
W + pneumococcus E ²	2,000	1,000	780	400	450
W (heated *) + pneumococcus E ²	2,200	1,500	3,400	∞	∞
W + pneumococcus B1 ³ †	2,750	2,200	4,050	∞	
Washed normal corpuscles 0.2 c.c. + normal serum + pneumococcus E	1,000	900	1,150	2,500	5,000
Washed normal corpuscles (heated *) 0.2 c.c. + normal serum 0.2 c.c. + pneumococcus E	1,050	1,300	2,000	∞	∞
Washed normal corpuscles 0.2 c.c. + NaCl sol. 0.2 cc + pneumococcus E	2,600	2,850	3,000	∞	∞

* 50° C. for 30 minutes.

† Virulent organism.

Table 10 shows the effect of normal defibrinated blood upon the pneumococcus. The early reduction in the number of colonies is more marked than in the sera. Occasionally sterile plates are obtained at the end of 24 hours. The table shows further that heating the blood or the washed corpuscles, using virulent strains of pneumococci which are not susceptible to phagocytosis, and adding

normal salt solution to the washed corpuscles, instead of serum, all diminish perceptibly the destructive effect.

TABLE II.
THE PNEUMOCOCCIDAL ACTION OF PNEUMONIC DEFIBRINATED BLOOD.

DEFIBRINATED PNEUMONIC BLOOD, 0.5 c.c.	DAY OF DISEASE	LEUCOCYTOSIS	COLONIES IN BLOOD AGAR PLATES				
			At Once	1.5 Hrs.	3 Hrs.	10 Hrs.	24 Hrs.
D + pneumococcus E ² ...	18 hours before crisis	21,000	2,800	400	12	6	0
D + " E ² ...	1st day after crisis	17,000	3,200	1,700	50	2	2,000
D + " E ² ...	10 days after crisis	0,000	1,890	2,100	3,500	4,000	5,000
P + " E ² ...	During crisis	27,600	2,460	1,500	400	30	2
P + " B I ⁵ ...	"	27,600	1,200	300	40	0	0
P + " E ² ...	4 days after crisis	10,000	3,640	4,200	3,500	∞	∞
S + " E ² ...	Crisis	23,000	2,300	1,000	4	25	0
S + " B I ⁵ ...	"	23,000	1,500	200	25	0	0
V + " S*	5th day of disease	10,500	45	215	3,400	∞	∞
Washed pneumonic blood 0.2 c.c. + pneumonic serum 0.2 c.c. + pneumococcus E ² ...	Day before crisis	21,000	3,000	800	50	30	20
Washed pneumonic blood (heated)† 0.2 c.c. + pneumonic serum 0.2 c.c. + pneumococcus E ² ...	Crisis	23,000	2,400	3,100	5,600	∞	∞
Washed pneumonic blood 0.2 c.c. + NaCl sol. 0.2 c.c. + pneumococcus E ² ...	"	23,000	2,700	2,400	4,500	∞	∞

* Virulent strain.

† Heated means 50° C. for 30 minutes.

Table II shows that the pneumococcidal effect of pneumonic defibrinated blood is, other things being equal, proportional to the leucocytosis and apparently bears no relation to the time the blood is withdrawn—before, during, or after crisis. This agrees with the findings¹ of Ruediger¹ in regard to the streptococcidal action of defibrinated blood. Furthermore, virulent strains grow very readily, and both living leucocytes and serum are necessary to bring about decrease in the number of colonies of non-virulent organisms.

Summary.—Normal and pneumonic sera have no pneumococcidal effect. The primary drop which occurs in serum cannot be looked upon as a true bacteriolytic action but as due rather to clumping or death of the organisms incident to change of environment. Occasionally samples of normal blood have a distinct pneumococcidal action which is due not, as first supposed, to individual differences, but to the number of leucocytes present. Pneumonic defibrinated blood is decidedly pneumococcidal, especially when the leucocytosis is high, 0.5 c.c. containing 50 millions of pneumococci may be rendered sterile in four hours.

¹ *Jour. Am. Med. Assoc.*, 1906, 46, p. 108.

These experiments seem to indicate that while neither leucocytes nor serum alone have any pneumococcal effect, their combination has a decided action, probably dependent on phagocytosis, and further experiments were made with a view of analyzing the mechanism involved.

TABLE 12.
PNEUMOCOCCAL ACTION OF HUMAN LEUCOCYTES.

MIXTURES	No. LEUCOCYTES PER CUBIC MILLIMETER	PHAGO- CYTOSIS 1 HOUR	No. COLONIES IN BLOOD AGAR PLATES			
			At Once	1.5 Hr.	10 Hrs.	24 Hrs.
Washed corpuscles 0.5 + fresh serum 0.5 . .	44,800	15	550	1	0	0
" " 0.5 + " " 0.5 . .	31,000	10	1,050	25	4	0
" " 0.5 + " " 0.5 . .	21,000	20	560	5	0	0
" " 0.5 + " " 0.5 . .	20,000	12	355	180	125	0
" " 0.5 + " " 0.5 . .	11,000	30	1,350	1,000	18	200
" " 0.5 + " " 0.5 . .	8,000	28	250	75	15	800
" " 0.5 + " " 0.5 . .	50	?	1,000	1,450	∞	∞
" " 0.5 + " " 0.5 . .	25	?	500	450	1,400	∞
Washed corpuscles (heated 50°C., 30 min.) 0.5 + fresh serum 0.5	44,800	0	700	625	4,300	∞
Washed corpuscles (heated 50°C., 30 min.) 0.5 + fresh serum 0.5	11,000	0	1,250	1,300	∞	∞
Washed corpuscles 0.5 + 0.85% NaCl sol. 0.5	28,000	0	375	320	2,400	∞
Washed corpuscles 0.5 + 0.85% NaCl sol. 0.5	8,000	0	1,300	1,350	∞	∞
Washed corpuscles 0.5 + fresh serum 0.5*	20,000	0	150	50	200	∞
Washed corpuscles 0.5 + heated serum (60°C. 1.5 H.)	5,500	8	1,450	1,200	1,850	1,600
Fresh serum	980	800	1,400	4,500

* Inoculated with highly virulent strain.

Table 12 is illustrative of results that have been obtained many times. The points which I wish to emphasize especially are, that the leucocyte counts were made from the tubes; that the degree of phagocytosis was obtained in parallel, but separate, tubes in which pneumococci were present in much larger numbers than in those used for study of the pneumococcal action. The reason phagocytosis is not so high when a large as when a moderate number of leucocytes was used is due to the fact that there were not enough pneumococci present, practically all having been taken up as shown by the smears. In order to obtain sterile plates it is necessary to use organisms which are freely taken up. Normal leucocytes and serum behave practically the same as pneumonic leucocytes and serum, although the former leucocytes are not quite so active as pointed out in the foregoing. While the experiments in Table 9 show that pneumonic serum alone has no appreciable pneumococcal effect I controlled each experiment given in Table 12 carefully

by inoculating serum, so I am sure that the destruction is not the result of something in the serum which would act independently of the leucocytes.

The results shown in Table 12 establish, I believe, that the pneumococcidal effect of pneumonic and other leucocytotic blood is due entirely to phagocytosis. The following points speak for this view: The destructive effect is most marked when the degree of phagocytosis is greatest. As phagocytosis is diminished by heating the leucocytes, by heating serum, or by adding falling quantities of serum, thus diminishing the opsonin, and by using virulent pneumococci, so the pneumococcidal action fluctuates. Other things being equal the destructive action is directly proportional to the number of healthy, well-preserved leucocytes present.

Definite evidences of digestion of pneumococci within leucocytes have been made out repeatedly. In experiments with a sufficiently large number of leucocytes so that destruction of pneumococci goes on more rapidly than multiplication, the picture is striking. Smears made at intervals show not only increasingly less pneumococci per leucocyte, but also morphologic and tinctorial evidences of solution (see Plate 22). This is seen best at the end of 8 to 12 hours.

CONCLUSIONS.

Non-virulent pneumococci are freely susceptible to phagocytosis. Virulent strains are not, but in the degree in which they lose their virulence when cultivated upon artificial media they become, susceptible to phagocytosis. When lost virulence is restored, resistance to phagocytosis also returns.

Pneumonic leucocytes are perceptibly more actively phagocytic than normal leucocytes, a property which seems independent of the action of the serum. They are more resistant to heat, and when heated to the thermal death-point and resuspended in serum, they are more actively chemiotactic for pneumococci.

Pneumococco-opsonin is quite resistant to heat. In pneumonia it may fall below normal in fatal cases, while during and shortly after crisis of cases that recover it seems to rise above normal.

Pneumococci when first isolated from the blood of pneumonia patients, whether before, during, or after crisis, refuse phagocytosis *in*

vitro, with only a few exceptions, whether exposed to the blood of the patient from which they are isolated or to blood from other sources.

The pneumococcidal action of pneumonic or other leucocytotic blood is the result of the combined action of serum and leucocytes—phagocytosis and intraphagocytic digestion. Dead leucocytes seem to have no pneumococcidal effect. Normal and pneumonic sera have no pneumococcidal action. The primary drop which often occurs cannot be looked upon as a true bacteriolysis, but as due rather to clumping or death of organisms incident to change of environment.

However important the rôle of phagocytosis in the destruction of pneumococci *in vitro*, there are still serious difficulties in the way of ascribing the crisis and healing of pneumonia to opsonification and phagocytosis alone. Thus definite experimental proof is yet lacking that the pneumococci that cause pneumonia absorb opsonin *in vivo* sufficiently to be made susceptible to phagocytosis.

I herewith wish to express my thanks to Dr. Hektoen for suggestions and aid in the work; and to the house and attending staffs of the Presbyterian Hospital for many courtesies.

EXPLANATION OF PLATE 22.

FIG. 1.—Phagocytosis of pneumococci by leucocytes in human defibrinated blood.

FIG. 2.—Attraction of pneumococci by heated leucocytes. Washed human blood was heated to 50° C. for 15 minutes, recombined with serum and placed in the shaking machine at 37° C. for 1 hour.

FIG. 3.—Digestion of pneumococci by human leucocytes in defibrinated blood. The suspension had been kept at 37° C. for 10 hours when the smear was made.

PLATE 22.

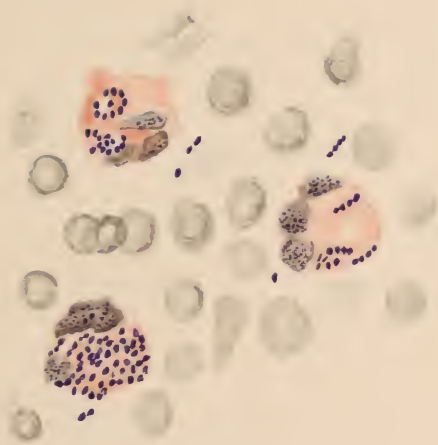


FIG. 1.

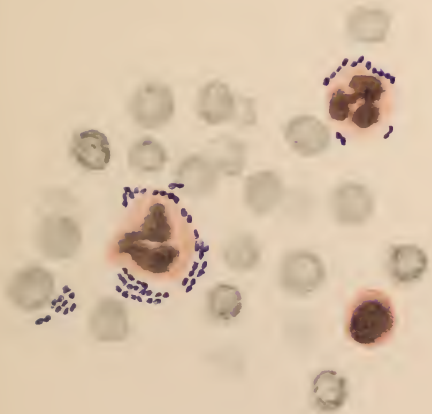


FIG. 2.

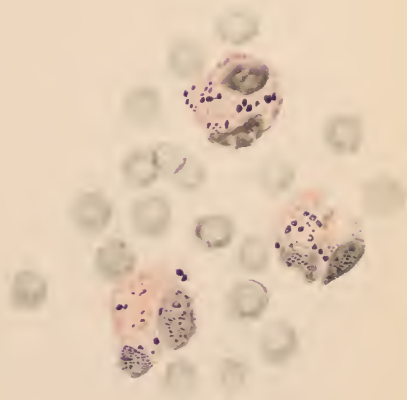


FIG. 3.

The Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 3

October 30, 1906

No. 5

THE ORAL ADMINISTRATION OF ANTITOXINS FOR
PREVENTION OF DIPHTHERIA, TETANUS, AND
POSSIBLY SEPSIS, WITH SOME OBSERVATIONS ON
THE INFLUENCE OF CERTAIN DRUGS IN PREVENT-
ING DIGESTION AND PROMOTING ABSORPTION
FROM THE ALIMENTARY CANAL.*

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At the the 1902 meeting of the American Medical Association at Saratoga Springs, N. Y., one of us presented a paper entitled: "The Absorption of Albumins and Globulins."¹ This paper gave a summary of the results obtained up to that time in attempting to find the conditions under which sera were absorbed from the alimentary canal and what agents, if any, could be used to further this result. The conclusion reached at that time was stated thus: "These experiments so far appear to show either that antitoxin is not absorbed in the same proportion as the total albumins of serum, or is changed in being absorbed, the majority of the guinea-pigs dying when injected with several times the fatal dose of toxin, despite the large absorption of albumins."

* Received for publication July 22, 1906.

¹ *Jour. Am. Med. Assoc.*, 1903, 40, p. 635.

Since that report, the work has been continued, and we are now able to take series after series of guinea-pigs or rabbits, and by the oral administration of antitoxin save 100 per cent of the treated animals, when the antitoxin is administered before the toxin; while the untreated ones, receiving the same dose of poison, invariably die. There were many failures before reaching this stage of the work. In many cases in the earlier work, one or two pigs of a series of 10 would survive, the rest die; but gradually we appeared to learn the conditions necessary for success and something also as to the medicinal agents which would assist in promoting absorption of the antitoxic serum. The work is far from being completed. We find that there are a number of medicinal agents, on the one hand, tending to arrest digestion, on the other, helping to promote absorption, which may be used; but as to which of these and in what quantities it is best to use, we are as yet unable to say with certainty.

One may well ask: As we know that antitoxic serum may be injected hypodermically with good results why use an uncertain method?

It appears to the writers that there are two principal reasons for the attempt: (1) there is a certain amount of danger from any and all hypodermic medication. Death has been reported from the injection of one-eighth of a grain of morphine. A number of sudden deaths have followed the injection of antitoxic serum. We mean now deaths not due to contaminated or impure serum, but apparently due to an idiosyncrasy of the patient, the fatal results following in a few moments or hours after the injection.

Again, there are a number of very unpleasant skin and joint complications, urticaria, erythema, purpura, angioneurotic edema, that may follow hypodermic injection of even small quantities of serum. So far it appears that these phenomena are more prominent in prophylactic than in curative injections. In the 17 men and boys used in the following experiments, not one has shown any skin or joint disturbance.

(2) A large part of the cost of antitoxic serum is due to the exceptional care, the repeated careful tests that must be made in order to secure the fitness of the material for hypodermic administration. Roughly speaking, antitoxic sera, as they come from the animal,

do not cost more than one-half to one-third of what the product ready for hypodermic injection actually costs. Again, it is more than probable, although not yet demonstrated, that the serum to be given per mouth can be given in the dry form, and in such condition it will keep indefinitely. The high cost of liquid serum limits, to a certain extent, the prophylactic use of the serum, and if we should be fortunate enough to find serum of value in preventing suppurative diseases, tuberculosis, and other chronic diseases, where it would be advisable to keep the patient under treatment for some time, there would manifestly be great gain in being able to give this material per mouth rather than hypodermically.

The results thus far obtained do not warrant the use of antitoxin per mouth for curative purposes. The results are, and probably always will be, more uncertain, and, as reported by several observers, the effect is much slower than by the hypodermic method.

Practically, if a child has been in school and tomorrow one of the pupils in the room with the child develops diphtheria, it is not known how severe the exposure has been, and one hesitates as to the desirability of giving the hypodermic injection of serum as a prophylactic, but, could one give one or two doses of serum per mouth at a moderate cost, the matter would take on a different light. Or, here is a minor or dirt-infected wound, is it worth while to fear tetanus, and give a prophylactic dose of serum hypodermically? The practical answer to this, given by the profession, often is to take the chance. The serum is not given.

In the voluminous literature on serum therapy many references are made to the oral administration of antitoxins. A few cases are reported of a certain amount of immunity being produced when the antitoxin was given or taken per stomach. Here and there some clinical observer has reported good results following such administration, but practically the entire consensus of opinion is that the oral administration of sera is without value. It is generally believed that both toxin and antitoxin are not absorbed as such. Our experiments, however, lead us to the conclusion that both toxin and antitoxin are absorbed as such, unless they are destroyed by the secretions of the alimentary canal.

The specific objects in view in this investigation are:

1. To confirm work already done, showing that under certain conditions antitoxins can be absorbed.
2. To find under what conditions antitoxins would be absorbed in the greatest amount and with the greatest uniformity.
3. To ascertain whether the oral administration of diphtheria and tetanus antitoxins can be depended upon as safe and advantageous prophylactic measures.

TECHNIQUE.

Guinea-pigs, rabbits, dogs, and men and boys have been used in the work thus far. In giving the antitoxin per mouth to guinea-pigs, several methods were tried. First, a small catheter was introduced into the stomach, and the serum was forced through this from a syringe or pipette. Later, the antitoxin was put into small capsules, and the pig was forced to swallow them. Finally it was found that the best way to administer the serum per stomach was to hold the mouth of the animal open with forceps, place the given amount of serum well back in the mouth very slowly, one drop at a time, and allow the pig to swallow it. By using care and patience and permitting the pig to close its mouth at intervals during the administration, in most cases not a drop of the serum was wasted.

Full-grown rabbits were treated by the above method very quickly and successfully. In case of small rabbits, the catheter sometimes had to be used.

The antitoxin was given to dogs by placing it in large gelatin capsules, and causing them to be swallowed by the dog.

ADMINISTRATION OF ANTIDIPHThERIC SERUM, ALONE AND IN COMBINATION WITH VARIOUS DRUGS, TO GUINEA-PIGS PER STOMACH

A series of 121 pigs was used, divided into groups of six, with one control pig for each group. The following drugs, in small amounts, were added to the serum given to some of the groups: trikresol, opium, sodium bicarbonate, morphine sulphate, and chloroform. Practically all of these pigs were lost, the administration of a few hundred units of antitoxin alone and in various combinations with the above drugs not giving sufficient protection against the injection of two or three fatal doses of toxin. Finally, however, it was found that pigs treated with serum to which morphine, cocaine, and trikresol had been added could be saved from a few fatal doses of toxin although with no very great degree of uniformity. These pigs were injected with the toxin 12 to 24 hours after the administration of the serum. Fifteen pigs treated in this way with from 1,000 to 2,000 units of antitoxin withstood from 2 to 10 fatal doses of toxin.

The results of these tests which we naturally concluded were based upon the paralyzing action of the morphine and cocaine upon digestion led us to try the administration of antitoxin per intestine.

The technique of administration per intestine consisted in making an abdominal incision, securing a loop of small intestine, and injecting into it with a very small needle a given amount of serum. The intestinal loop was then replaced and the peritoneum and skin sewed up. Ether was used as the anesthetic.

The following series of pigs were treated with diphtheria antitoxin per intestine, and 15 hours after were injected subcutaneously with the toxin:

TABLE I

Guinea-Pig No.	Units Anti-toxin	Fatal Doses Toxin	Result
1.....	600	5	Dead 6 days*
2.....	300	5	+
2.....	600	5	+
4.....	300	5	+
5.....	300	5	+
6.....	600	10	+
7.....	600	10	+
8.....	300	5	Dead 4 days*
9.....	300	10	+
10.....	300	15	+
11.....	300	20	+
12.....	300	25	Dead 3 days
13.....	300	35	+
14.....	300	40	+
15.....	300	50	Dead 5 days
16.....	300	60	" 5 "
17.....	300	70	" 5 "
18.....	300	25	" 3 "
19.....	300	35	" 3 "
20.....	300	40	+
21.....	300	50	+
22.....	300	60	+
23.....	300	70	Dead 3 days
24.....	300	25	+
25.....	300	35	+
26.....	300	40	+
27.....	300	50	Dead 4 days
28.....	300	60	" 3 "
29.....	300	70	" 4 "
30.....	300	80	" 3 "
31.....	300	25	" 4 "
32.....	300	35	+
33.....	300	40	Dead 3 days
34.....	300	50	" 4 "
35.....	300	60	" 3 "
36.....	600	40	" 2 "
37.....	900	50	+
38.....	1,500	70	+
39.....	2,100	100	Dead 2 days
40.....	600	20	+
41.....	900	25	+
42.....	1,500	35	Dead 4 days
43.....	1,800	40	+
44.....	2,100	45	+
45.....	2,100	45	Dead 6 days
46.....	2,400	50	+
47.....	300	15	+
48.....	300	20	+
49.....	200	25	+
50.....	300	25	+
51.....	300	30	Dead 3 days
52.....	300	35	" 3 "
53.....	300	40	+

With this series of 53 pigs 11 control pigs untreated and injected with 5 fatal doses of toxin, all died in from 2 to 4 days. Pigs 1, 8, and 16 died from peritonitis. It is possible that others died from shock.

Taking into consideration the delicacy of the operation and danger of death from shock and peritonitis, the results are fairly uniform, 30 pigs being saved out of the 53. It will be observed that many of these received enormous doses of toxin and comparatively small administrations of antitoxin.

Most of the pigs in the above series were given, per intestine, antitoxin to which was added small amounts of strychnin and potassium bicarbonate.

The result of the work in administering antitoxin per intestine demonstrates that absorption from the intestine and consequently protection from relatively enormous doses of toxin is possible. Our efforts were now directed toward administering the antitoxin orally and producing conditions such that the antitoxin would be unaffected by peptic digestion and pass into the intestine for the most part unchanged.

ORAL ADMINISTRATION OF ANTIDIPHThERIC AND ANTITETANIC SERA IN COMBINATION WITH VARIOUS DRUGS.

The specific purpose at this time in our work was to protect the antitoxin from peptic digestion, and cause it to pass into the intestine unchanged. A series of 104 pigs was used, divided into groups of 4 to 6, with a control pig in each group. Each pig received per stomach from 300 to 1,500 units of antidiphtheric serum in combination with various drugs. Fifteen hours after giving the serum each pig received five fatal doses of toxin subcutaneously. The following substances alone and in various combinations were added to the serum: cocaine, morphine, calomel, strychnin, opium, sodium bicarbonate, potassium bicarbonate, bismuth subnitrate, oil emulsion, and soap solution. Every one of these 104 pigs was lost.

Finally, after experimentation with several series of pigs, it was found that a combination of fluid opium and a saturated solution of salol in chloroform, added to the serum, and the mixture thoroughly shaken until an emulsion was produced, gave the best results. The

serum in all cases contained 0.04 c.c. of 1 per cent trikresol. The saturated solution of salol in chloroform and fluid opium were added in the proportion of one drop of the former to each c.c. and one drop of the latter to each 10 c.c. of antitoxin. The fluid opium concentrated contained 22 grains crystallized morphine in each fluid ounce, and was four times stronger than Tincture Opium U. S. P. Hereafter in this work all antitoxin given per mouth was prepared with the above ingredients.

Out of a series of 61 pigs treated by this method, 57 were saved, or 93.4 per cent. These pigs were kept without food for 24 to 48 hours before treatment, and 12 to 24 hours after administration of the serum were injected subcutaneously with the toxin; 15 control pigs, receiving the same amount of toxin as those treated, all died in less than five days.

The following table gives the result of this series with the amount of antitoxin and dosage of toxin used:

TABLE 2.

Group	No. Units Antitoxin	No. Fatal Doses Toxin	No. Pigs in Group	No. Control Pigs in Group	No. Treated Pigs in Group	No. Treated Pigs Lost	No. Treated Pigs Saved
44.....	600	3	4	1	3	0	3
45.....	1,200	3	6	1	5	0	5
48.....	1,200	3	6	1	5	1	4
49.....	1,000	3	12	2	10	0	10
52.....	1,500	3	10	2	8	0	8
54.....	1,500	3	10	2	8	2	6
55.....	1,500	2	6	2	4	0	4
56.....	1,500	2	8	2	6	0	6
57.....	1,500	2	12	2	10	1	9
Total...			74	15	59	4	55

Diphtheria antitoxin per stomach, rabbits.—Eighteen rabbits in groups of three were treated with antidiphtheric serum. With the exception of two groups which were injected with overwhelming doses of toxin in proportion to the high susceptibility and low resistance of rabbits to diphtheria, every animal was saved. In other words, out of 12 rabbits treated with 600 to 1,500 units of serum and afterward injected with one fatal dose of toxin for each 250 gr. weight, 100 per cent were saved. We feel safe in stating that, with proper care in treating and handling, rabbits can be saved from toxic doses of diphtheria by the oral administration of a reasonably small amount of antitoxin, without the loss of a single animal. All

control rabbits, injected with the same amounts of toxin as the treated ones, died in from two to four days. The results are given in the table below.

TABLE 3.

Rabbit No.	Weight Grams	Units Antitoxin	Fatal Doses Toxin	Result
1.....	1,855	Control	7.6	Dead 3 days
2.....	2,585	1,500	12.3	+
3.....	1,760	900	7.2	+
4.....	1,040	600	7.9	+
5.....	2,180	Control	10.7	Dead 2 days
6.....	2,585	600	12.3	3
7.....	2,115	450	10.4	+
8.....	1,790	300	9.1	Dead 3 days
9.....	1,500	Control	8.0	3
10.....	1,815	450	9.26	" 3 "
11.....	1,800	600	9.2	" 2 " (pregnant)
12.....	1,420	900	7.68	+
13.....	1,330	Control	6.32	Dead 3 days
14.....	1,015	900	5.06	+
15.....	1,375	900	6.5	+
16.....	2,060	900	9.24	+
17.....	580	Control	4.2	Dead 3 days
18.....	725	1,500	4.9	+
19.....	890	1,500	5.5	+
20.....	825	1,500	5.3	+
21.....	3,150	Control	9.0	Dead 2½ days
22.....	2,200	1,500	8.8	+
23.....	2,000	1,500	8.0	+
24.....	2,700	1,500	9.0	+

Diphtheria antitoxin per mouth, dogs.—Nine dogs, indicated in the following table, after treatment with the antitoxin, were injected with one fatal dose of toxin for each 500 to 750 gr. weight. Dogs 7 to 9 were injected with one fatal dose for each 500 gr. weight plus three fatal doses for each dog. All in this group died; however, two of them lived 7 and 8 days, while the control died in 4 days. Dog 5 was sick with distemper before treatment. Therefore, exclusive of the group of dogs which received the overwhelming doses of toxin, practically 100 per cent of the treated dogs lived.

TABLE 4.

Dog Number	Weight Grams	Units Antitoxin	Fatal Doses Toxin	Results
1.....	11,580	Control	15.4	Dead 7 days
2.....	7,370	4,500	9.8	+
3.....	7,870	4,837.5	10.5	+
4.....	5,600	Control	11.2	Dead 3 days
5.....	7,000	6,000	14.0	" 6 " (distemper)
6.....	7,020	Control	17.0	" 4 "
7.....	9,000	6,000	21.0	" 7 "
8.....	10,390	6,000	23.7	" 8 "
9.....	11,000	6,000	25.0	" 4 "
10.....	11,580	Control	15.4	" 7 "
11.....	14,000	9,000	17.0	+
12.....	9,700	9,000	13.0	+
13.....	11,400	9,000	15.3	+

Tetanus antitoxin per stomach, guinea-pigs.—In all, 97 pigs were used in testing the absorption of tetanus antitoxin. The table below gives those from the sixth group on. Many of those in the first groups were saved, but not until tests were made on several pigs was the correct dosage of antitoxin and toxin regulated, so that uniform results followed. The most of the pigs received administrations of serum on two or three successive days, in the meantime being kept without food. For instance, 10 units were given the first day, 10 units the second day, and the injection of the toxin was made on the second or third day.

TABLE 5.

Group	No. Units Antitoxin	No. Fatal Doses Toxin	No. Pigs in Group	No. Control Pigs in Group	No. Treated Pigs in Group	No. Treated Pigs Lost	No. Treated Pigs Saved
6.	20	2	5	1	4	1	3
7.	60	2	3	1	2	0	2
8.	80	3	3	1	2	0	2
9.	50	3	10	2	8	0	8
10.	40	2	6	2	4	0	4
11.	40	2	5	1	4	1	3
12.	30	2	3	1	2	0	2
13.	40	2	6	1	5	0	5
14.	40	2	5	1	4	0	4
Total.....			46	11	35	2	33

Four of the above pigs, which are indicated as saved, showed symptoms of tetanus and recovered. All of the 11 control pigs died with tetanic spasms in less than eight days after injection. Ninety-four and two-sevenths per cent of the treated pigs were saved.

In Group 10, indicated above, four additional pigs were treated with 40 units of antitoxin exactly as the four pigs which, as shown in the table, were saved. The two fatal doses of toxin, however, instead of being injected after treatment, as in the case of the four pigs above, were injected just before administering the first 10 units of antitoxin with the following results:

G.-pig 7, symptoms 3 days, dead 5 days; G.-pig 8, slight symptoms 5 days, recovery; G.-pig 9, no symptoms; G.-pig 10, symptoms 3 days, dead 6 days.

This experiment strengthens the clinical observations that sufficient time must be allowed for absorption of antitoxin when given per mouth.

The following group of pigs received the toxins, to which were added chloroform, salol, trikresol and opium, in one administration:

G.-pig	1,	30	fatal doses	diphtheria toxin	Dead 7 days
"	2,	60	"	"	"	"	"	"	"	"	3 "
"	3,	90	"	"	"	"	"	"	"	"	+
"	4,	15	"	"	tetanus	"	"	"	"	"	+
"	5,	30	"	"	"	"	"	"	"	"	Sick 3 days, recovered
"	6,	60	"	"	"	"	"	"	"	"	Dead 4 "

As the toxins used in the two groups above had been diluted with a small amount of beef broth (1 per cent peptone), it was suggested that possibly the pigs died from absorption of peptone (albumoses). Wishing also to eliminate the possibility of death resulting from the administration of the drugs in the prepared toxins, the group below was treated per mouth as follows:

G.-pig	1,	$\frac{1}{2}$	c.c. broth to which the drugs were added	+
"	2,	1	"	"	"	"	"	"	"	"	+
"	3,	1	"	"	"	"	"	"	"	"	+
"	4,	1	broth, drugs not added	+
"	5,	1	broth, " " "	+
"	6,	1	distilled water to which the drugs were added	+
"	7,	1	"	"	"	"	"	"	"	"	+

In the above group the administrations were made in one dose and in three doses, as in the experiment where the toxins were given. The drugs were added in exactly the same proportion as were added to the toxins.

The following pigs received the toxins, to which the drugs were added, per mouth in three administrations, and before treatment were injected with relatively large amounts of the respective antitoxins:

G.-pig	1,	60	fatal doses	tetanus toxin	+
"	2,	60	"	"	"	"	"	"	"	"	+
"	3,	60	"	"	"	"	"	"	"	"	+
"	4,	60	"	"	"	"	"	"	"	"	+
"	5,	60	"	"	"	"	"	"	"	"	+
"	6,	100	"	"	"	"	"	"	"	"	+
"	7,	100	"	"	"	"	"	"	"	"	+
"	8,	90	"	"	diphtheria toxin	+
"	9,	90	"	"	"	"	"	"	"	"	+

The following group of pigs received the solutions per intestine. No drugs were added to the toxins.

G.-pig	1,	10	fatal doses	tetanus toxin	+
"	2,	50	"	"	"	"	"	"	"	"	+
"	3,	100	"	"	"	"	"	"	"	"	+
"	4,	200	"	"	"	"	"	"	"	"	Sick 3 days, dead 6 days
"	5,	1	c.c. 1 per cent peptone	broth	+
"	6,	5	c.c. 1	"	"	"	"	"	"	"	+

Gr.-pig	7,	200	fatal	doses	tetanus	toxin	with	drugs	.	.	Died	from	operation
"	8,	100	"	"	diphtheria	"	"	"	.	.	Dead	2	days
"	9,	100	"	"	"	"	"	normal	horse	serum	+		
"	10,	100	"	"	tetanus	"	"	"	"	"	+		
"	11,	200	"	"	"	"	"	"	"	"	+		
"	12,	1	c.c.	normal	horse	serum	+	
"	13,	2	c.c.	"	"	"	+	
"	14,	100	fatal	doses	tetanus	toxin	with	carbonated	water		+		
"	15,	100	"	"	"	"	"	"	"	"	+		

TETANUS ANTITOXIN PER STOMACH, RABBITS AND DOGS.

Experiments have been conducted upon a series of 20 rabbits with tetanus antitoxin, but up to the present neither positive nor negative results have been established. This is due to the failure thus far to find the correct amount of tetanus toxin to kill, without giving overwhelming doses. Symptoms have been produced in control rabbits, however, while treated rabbits of the same group have shown no symptoms.

Owing to the naturally high resistance of dogs to tetanus toxin, similar work with them has not been satisfactory. Several dogs have been experimented upon, none of which showed symptoms after the injection of enormous doses of toxin. It is interesting to note that one dog, weighing 15 kilos, has shown no symptoms after the injection of enough tetanus toxin to kill 1,270 guinea-pigs.

ABSORPTION OF DIPHTHERIA ANTITOXIN FROM ALIMENTARY CANAL OF DOGS.

Thus far the absorption of diphtheria antitoxin has been tested on four dogs. These dogs were starved for 24 hours previous to the operation, and the stomach emptied with apomorphine. The abdomen was opened, and the stomach tied off at the pylorus. Three sections of the intestine were used; one in the jejunum below the opening of the pancreatic duct; another at the end of the ileum; and the other in the rectum. The intestinal loops were 11 cm. long, their mesenteric attachments were unimpaired, rough handling was avoided, and, after the introduction of the serum, the organs were returned to the abdomen, the opening closed and the abdomen kept warm. After the serum had been in the organs for one hour, the amount remaining was determined by precipitating the remaining albumin, drying and weighing.

To the serum used in these experiments, trikresol, chloroform,

salol, or opium were added. The results are given in the appended table:

TABLE 6.

Antitoxin Mixture Diluted with Saline Solution	Stomach	Jejunum	Ileum	Rectum
Dog I. General Anesthesia—				
Percentage by volume of mixture absorbed..	Excess	14.3%	42 ⁸ / ₁₀₀ %	Excess
weight of albumins	0	0	12 ¹ / ₂	0
Number of antitoxic units injected.....	3,955	396	396	396
absorbed.....	None	None	49	None
Dog II. Local Anesthesia—				
Percentage by volume of mixture absorbed..	Excess
weight of albumins	12 ³ / ₁₀₀ %
Number of antitoxic units injected.....	2,825
absorbed.....	57
Dog III. Local Anesthesia—				
Percentage by volume of mixture absorbed..	62 ¹ / ₁₀₀ %	68 ¹ / ₁₀₀ %
weight of albumins	25	12 ¹ / ₂
Number of antitoxic units injected.....	452	452
absorbed.....	113	56
Antitoxin Mixture Not Diluted	Stomach	Jejunum	Ileum	Rectum
Dog IV. Local Anesthesia—				
Percentage by volume of mixture absorbed..	Excess	18%
weight of albumins	52.3%	28 ¹ / ₂
Number of antitoxic units injected.....	2,763	2,763
absorbed.....	1,430	802

EXPERIMENTS WITH DIPHTHERIA AND TETANUS ANTITOXINS PER STOMACH, HOMO.

In conducting the following experiments the individual under treatment was bled from the median cephalic vein either before treatment with the antitoxin or a few weeks afterward. The serum was collected from this blood and, together with a given number of fatal doses of the toxin, was injected subcutaneously into guinea-pigs.

Each person swallowed a given amount of prepared antitoxin, in most cases on two or three successive days and the blood was again collected as before and tested as to its protective influence against the toxin in question, by injecting guinea-pigs with the blood serum and the toxin. A comparison of these results with those from injections of the human blood serum, before the individual took the antitoxin per mouth, indicated whether the diphtheria or tetanus antitoxin, which had been administered to the individual, had resisted digestion and had been absorbed in sufficient quantities to cause the protective antitoxin to be present in the blood of the individual.

The appended table illustrates the effect of the oral administration of the prepared serum *per os*.

TABLE 7.

DIPHTHERIA AND TETANUS ANTITOXINS, PER MOUTH, HOMO				GUINEA-PIG INJECTIONS BEFORE AND SEVERAL WEEKS AFTER ADMINISTRATION OF ANTITOXIN TO SHOW THAT THE HUMAN SERUM CONTAINED NO NATURAL PROTECTION ALL PIGS KILLED BY TOXIN						GUINEA-PIG INJECTIONS A FEW HOURS AFTER ADMINISTRATION OF ANTITOXIN, SHOWING PROTECTION IN BLOOD OF INDIVIDUAL. ALL PIGS LIVED					
Case	Sex	Age	Kind of Antitoxin Administered	Before Treatment		After Treatment			No. c.c. Serum	Fatal Doses Toxin	No. c.c. Serum	No. Fatal Doses Toxin	Time after Treatment Injec. Was Made	No. Units Antitoxin in 1 c.c. Blood of Individual Treated	No. Units Antitoxin in Total Quantity Blood
				No. c.c. Serum	No. Fatal Doses Toxin	No. c.c. Serum	No. Fatal Doses Toxin	No. Weeks after Taking Antitoxin							
1. W. E. K.	Male	20	Diph.	1 c.c.	5	2 c.c.	3	5 weeks	1 c.c.	5	1 c.c.	5	72 hrs.	0.05	283.7
2. W. K.	"	15	"	1	5	2	3	5 "	1	5	1	5	72 "	0.028	128.3
3. L. T. C.	"	24	"	2	3	5 "	2	5	2	5	6 days	0.025	140.3
4. E. S.	"	7	"	2	5	6 "	2	10	2	10	82 hrs.	0.05	181.5
5. E. H.	"	16	"	2	7	7 "	2	10	2	10	5 days	0.05	180.0
6. E. H. H.	"	20	"	2	4	3 "	2	5	2	5	24 hrs.	0.015	84.0
7. R. S.	"	7	"	2	5	3 "	2	7	2	7	24 "	0.116	421.0
8. W. P.	"	7	"	2 c.c.	3	1	5	1	5	0 days	0.07	245.0
9. H. K.	"	10	"	2	1	5	1	5	72 hrs.	0.05	300.0
10. W. E. K.	"	20	Tet.	2	5	2	3	2	3	72 "	0.000015	0.0084
11. B. P.	"	5	"	2	3	2	4	2	4	48 "	0.000002	0.008
12. W. K.	"	15	"	2	3	2	3	2	3	72 "	0.000003	0.0060
13. E. H.	"	16	"	1.5	3	1.5	3	1.5	3	0 days	0.000045	0.01783
14. L. T. C.	"	24	"	2	2	2	2	2	2	60 hrs.	0.000001	0.005075

One additional case is still under observation. No experiment has been omitted, and thus far all the cases treated per mouth have given positive results. We believe the results to be trustworthy and accurate, because check pigs, in making injections of human serum and toxins, have been used in all cases.

Reference to the table on preceding page will show that in some cases the human serum apparently possessed some protection before taking the antitoxin or several weeks after taking it. Thus, in Case 9, 2 c.c. of the blood serum, before giving the antitoxin, protected against the diphtheria toxin up to five fatal doses. Five fatal doses killed. In Case 5, 2 c.c. of the serum protected against the toxin up to seven fatal doses. Seven fatal doses killed. This is in accordance with the observations of Wassermann,¹ who has found in normal human serum some protection against diphtheria toxin in a certain percentage of cases.

It will be observed, however, that, in those cases where some natural protection seemed to exist, as well as in those whose blood serum contained no protection against the toxin, a few hours or a few days after administering the antitoxin the blood serum exerted considerable more protective influence.

It is clearly possible that more antitoxin would have been absorbed from the alimentary tract in this series of experiments, had the serum been given on an empty stomach. Experiments on guinea-pigs, and rabbits certainly indicate that moderate starvation before administration of the antitoxin is of considerable aid in promoting absorption.

EXPERIMENTS TO DETERMINE AMOUNT OF DIPHTHERIA ANTITOXIN ABSORBED FROM SUBCUTANEOUS INJECTION.

Experiment 1. Dog.—Injected subcutaneously with 800 units of antidiphtheric serum. Forty-eight hours after, collected 40 c.c. blood from superficial epigastric artery. Weight of dog 12,620 gr. One-twelfth body weight = 1,050 c.c. blood in body of dog.

Supposing that all of the injected antitoxin appeared in the blood stream, there should have been 800 units antitoxin in 1,050 c.c. of blood, or 0.761 units in 1 c.c. One c.c. serum ought to have protected against 76.1 fatal doses of diphtheria toxin.

¹ *Zeitschr. für Hyg.*, 1895, 19, p. 408.

This series of experiments is sufficient to show that, even with the subcutaneous injection of diphtheria antitoxin, only a portion of it appears in the blood.

We were not able to make a direct comparison of the results obtained by the two methods of administration of the antitoxin to homo. It will be noticed in the table illustrating the effect of oral administration, under column headed "Guinea-pig injections a few hours after administration, etc.," that the pigs withstood from 5 to 10 fatal doses of toxin, in case of diphtheria, when injected together with 2 c.c. of the human serum. However, in all but three of the cases treated with diphtheria antitoxin, the maximum amount of toxin that could be injected was not determined, owing to the difficulty of obtaining sufficient blood. Therefore, the maximum amount of antitoxin present in the blood of those cases treated with the antitoxin per mouth was not determined in all cases. Moreover, from these experiments it was clearly shown that a large amount of antitoxin given per mouth was not absorbed in proportion to the amount given, a reasonable amount of serum producing just as good protection.

None of those cases, included in our experiments in which the antitoxin was injected subcutaneously, received more than 1,040 units of serum, while some of the cases which were given the antitoxin per mouth received several thousand units. Experiment seemed to show conclusively that 3,000 units afforded as much protection as 6,000 or 13,000 units.

Taking these facts into consideration, and assuming that the oral administration be used only as a prophylactic, we believe the results obtained from administration per mouth compare very favorably with those obtained from subcutaneous injection. In the case of children, we find that the antitoxin is absorbed uniformly and in sufficient amount to give protection, when administered per mouth.

SENSITIZING ACTION OF HORSE SERUM UPON GUINEA-PIGS.

The recent publication by Rosenau and Anderson¹ of the Public Health and Marine Hospital Service, dealing with a newly identified poisonous action of blood serum, suggested to us the following experiment on guinea-pigs. Concerning the sensitization of guinea-pigs by

¹ No. 29. Pub. H. & Mar. Hosp. Service, *Hygienic Lab. Bull.*, 1906.

administration of horse serum per stomach, the authors concluded that "guinea-pigs may be sensitized to the toxic action of horse serum by feeding them with horse serum or horse meat." Nevertheless their work shows that guinea-pigs do not appear to be nearly so susceptible to the toxic action of horse serum when given per stomach as when injected subcutaneously or intraperitoneally. Out of a series of 38 pigs, which were given per mouth relatively enormous quantities of horse serum by Rosenau and Anderson, but two died.

The following series of pigs illustrates the comparative sensitizing action of reasonable amounts of horse serum, when injected and when given per stomach to guinea-pigs.

G.-pig 1, control

" 2,	$\frac{1}{250}$	c.c. antidiph. serum, injected	subcutaneously
" 3,	$\frac{1}{250}$	" "	" "
" 4,	$\frac{1}{250}$	" "	" "
" 5,	$\frac{1}{250}$	" "	" "
" 6,	$\frac{1}{1000}$	" "	" "
" 7,	$\frac{1}{1000}$	" "	" "
" 8,	$\frac{1}{2}$	" "	" "
" 9,	1	" "	" "
" 10,	1	" "	" "

Thirteen days afterward these pigs were treated as follows:

G.-pig 1, 6 c.c. antidiph. serum, injected into peritoneum	No symptoms
G.-pig 2, 6 antidiph. serum, injected into peritoneum	Symptoms 5 min., recovery
G.-pig 3, 6 antidiph. serum, injected into peritoneum	" 3 " "
G.-pig 4, 6 antidiph. serum, injected subcutaneously	" 40 " "
G.-pig 5, 6 antidiph. serum, injected subcut.	" 25 " "
" 6, 6 " " " intraper.	" 3 min., dead 40 min.
" 7, 6 " " " subcut.	" 6 min., recovery
" 8, 6 " " " "	" 15 " "
" 9, 6 " " " "	No symptoms
" 10, 6 " " " intraper.	Symptoms 1 " "

Another series of 10 pigs was treated per stomach with the same amounts of serum and at the same times as the series above. None of these showed any symptoms from the second administration of serum.

A series of experiments was undertaken to determine the effects of artificial peptic digestion on diphtheria antitoxin. The results agreed entirely with those heretofore reported, namely, the antitoxin is destroyed by peptic digestion.

SUMMARY AND CONCLUSIONS.

1. Thus far our experiments to prevent or cure sepsis by the oral administration of sera, do not allow us to draw any conclusion as to their value. The experiments are being continued, and will be reported on later.

2. So far we have been able to show that the antitoxins for diphtheria and tetanus may be given per mouth and absorbed in sufficient quantity to show markedly antitoxic properties in the blood of the treated animal. This result has been obtained from the use of certain drugs with the serum: on the one hand, drugs that inhibit digestion; on the other, those that promote absorption. It remains to be shown what are the best drugs for this purpose and in what quantities. (To this end our work is being continued as rapidly as possible.)

3. The results of the experiments, showing that the toxins of diphtheria and tetanus are absorbed from the alimentary canal, provided digestion is inhibited, we believe to be noteworthy. It suggests a method for slow, gradual immunization of animals or men, that is at the same time much easier and safer than hypodermic administration of toxin. It also suggests an explanation of many cases of toxemia, due to the absorption of putrefactive products where digestion is deranged.

4. When antidiphtheric serum alone is given to guinea-pigs per stomach, absorption of the antitoxin does not take place with any degree of uniformity.

5. Antidiphtheric serum alone given to guinea-pigs per intestine is absorbed in relatively large quantities. Small amounts of strychnin and potassium bicarbonate, added to the serum given per intestine, aids absorption to some extent.

6. Diphtheria antitoxin to which trikresol, salol, chloroform, and opium are added, when given to guinea-pigs, rabbits, and dogs per stomach, is absorbed in considerable amount and with reasonable uniformity.

7. Tetanus antitoxin, similarly prepared, administered to guinea-pigs per mouth, is equally as well absorbed.

8. Several hours must be allowed for absorption of antitoxin given per mouth.

9. Moderate starvation before and during administration of anti-toxin per mouth enhances absorption.

10. Large amounts of tetanus and diphtheria toxins, administered alone per mouth, are rarely absorbed.

11. Preliminary work seems to show that tetanus and diphtheria toxins, which are prepared with chloroform, salol, trikresol, and opium, given per mouth, are absorbed with some degree of uniformity.

12. Diphtheria antitoxin placed in the stomach and different portions of the intestine of dogs, for a period of one hour, is absorbed in considerable quantities.

13. Diphtheria and tetanus antitoxins, to which trikresol, salol, chloroform, and opium have been added, given to human beings per mouth, are absorbed in sufficient quantities to show protective qualities in the blood serum of the individual.

14. When diphtheria antitoxin is injected subcutaneously into man, dogs, rabbits, and guinea-pigs, only a part of it appears in the blood.

15. The sensitizing action of horse serum, given to guinea-pigs per mouth is not nearly so great as when injected subcutaneously or into the peritoneum.

In conclusion we wish to express our sincere thanks to Dr. W. M. Donald for aid in obtaining clinical material in carrying out the experiments upon human subjects.

PHAGOCYTOSIS OF RED CORPUSCLES.*

LUDVIG HEKTOEN.

(From the Memorial Institute for Infectious Diseases, Chicago.)

SAVTCHENKO,¹ Levaditi,² Gruber³ all observed that injection of serum of rabbits immunized with guinea-pig blood into the peritoneal cavity of guinea-pigs is followed by a marked erythrophagocytosis not only in the abdomen, but also in the blood-making organs, especially the spleen, and even in the circulating blood (Levaditi). In this phagocytosis take part microcytes as well as macrocytes. Ruziczka noted the occurrence of phagocytosis *in vitro* of red corpuscles in the presence of the corresponding immune serum.⁴ This phagocytosis was ascribed by Savtchenko to the action upon either the phagocytes or the erythrocytes of the amboceptor or *substance sensibilisatrice* in the immune serum, and this explanation is not contested by either Levaditi or Gruber. A year ago Neufeld and Töpfer⁵ showed that the blood of rabbits immunized with goat blood contains a substance that by action on goat corpuscles after absorption by them renders them subject to phagocytosis by guinea-pig leucocytes *in vitro*. This substance, which they designated as hemotropic, had no direct action on the leucocytes. Elsewhere⁶ I have pointed out that such substances should be called opsonins (hemopsonins) because of their analogy to the bacteriopsonins of Wright and Douglas who were the first to show clearly the opsonic function of the serum in phagocytosis of bacteria. Barratt⁷ found that doves immunized with hen blood give a serum that is strongly opsonic for hen corpuscles and demonstrated that in this, as well as other cases, the immune serum acts upon the erythrocytes and not directly upon the phagocytes. Neufeld and Töpfer, as well as Barratt conclude that the hemotropic substances, or hemopsonins, are distinct

* Received for publication July 1, 1906.

¹ *Ann. de l'Inst. Past.*, 1902, 16, p. 106.

² *Ibid.*, p. 233.

³ *Wien. klin. Wchnschr.*, 1903, 16, p. 1097.

⁴ See Gruber, *loc. cit.*

⁵ *Centralbl. f. Bakt.*, 1905, Orig. 37, p. 456.

⁶ *Jour. Am. Med. Assoc.*, 1906, 46, p. 1407.

⁷ *Proc. Roy. Soc., B.* 1905, 76, p. 524.

from the amboceptors and agglutinins, because a serum may be lytic or agglutinating, but not opsonic, and vice versa, with respect to certain erythrocytes; and recently I have shown that the specific amboceptors in an immune serum (serum of rabbits immune to goat blood) may be separated from the specific opsonins by absorption methods.¹ Keith² also has shown that hemopsonins are distinct from hemolytic amboceptors.

Barratt found immune opsonins to be quite resistant to heat and I have observed that heating to 70°C. for one hour the serum (diluted by one-half with m/8 NaCl solution) of rabbits immune to goat blood had no pronounced effect upon its opsonic power. Immune sera retain their hemopsonic power for months when kept at 3°-4° above 0° C.

TECHNIQUE.

Phagocytosis of red corpuscles *in vitro* may be studied in the following manner:

In case the serum in question is lytic for the corpuscles employed, which is commonly the case with immune serum, i. e., serum of an animal injected several times with increasing doses of alien blood,* then it is necessary first to destroy the hemolytic complement by heating at say 60° C. for 30 minutes in order to prevent laking. So far as my experience goes heating in this manner has no appreciable effect upon the hemopsonins. A small quantity of serum, e. g., 0.1 c.c. is now mixed with 0.1 or 0.2 c.c. of a 5 per cent suspension in NaCl solution of washed red corpuscles, and to this is added a small quantity of washed leucocytes contained in citrated or defibrinated blood or obtained from fresh aleuronat exudates, and the mixture placed at 37° C. for 60 minutes or so when smears are made and stained. When the serum, the erythrocytes, and the leucocytes employed are homologous the heating of the serum is unnecessary. If the serum is actively opsonic for the erythrocytes and the leucocytes phagocytically active, many of these will be found to be packed with unchanged red corpuscles. Laking in the ordinary sense does not occur, but after some time the intraleucocytic corpuscles may swell up or coalesce to form quite large globules. Further than this I have been unable to trace the fate of the corpuscles ingested by leucocytes *in vitro*. In many instances leucocytes of various species may be used as phagocytes, and one is not necessarily limited for this purpose to the leucocytes of the species furnishing the serum. Thus the serum of rabbits immune to goat blood may subject corpuscles to phagocytosis by dog, guinea-pig, human, and other leucocytes.

¹ *Jour. Infect. Dis.*, 1906, 3, p. 434.

² *Proc. Roy. Soc., B.*, 1906, 77, p. 537.

* In rabbits immune hemopsonins develop in response to injections of goat serum, goat corpuscles washed repeatedly in NaCl solution, and the washed stroma of laked corpuscles.

The extent to which immune serum is specific in its hemopsonic powers does not seem to have received much consideration as yet. Naturally one would expect to find that immune hemopsonin like the hemolytic amboceptor and hemagglutinins may find suitable receptors in other corpuscles than the particular kind used for immunization. Indeed, experiments with serum of rabbits immunized to goat corpuscles shows this supposition to be correct: 0.1 c.c. of this serum (heated to 60° C. for 30 minutes) has been found to opsonize, for phagocytosis by washed dog leucocytes, not only goat corpuscles, but also the corpuscles of sheep, dog, rabbit, chicken, pigeon, guinea-pig, and man. Using guinea-pig leucocytes as phagocytes the range of opsonic action in the same quantity of immune serum is limited almost exclusively to goat and sheep corpuscles. Using falling quantities of the immune serum and dog leucocytes as phagocytes it is found that the opsonic effect becomes limited to goat and sheep corpuscles, the approximate minimum opsonic dose for which has been found to be 0.0125 c.c. under the conditions of these experiments. These facts are set forth in Table I.

PHAGOCYTOSIS OF DIVERSE CORPUSCLES UNDER THE INFLUENCE OF SERUM OF RABBITS,
(1) IMMUNE TO GOAT BLOOD AND (2) NORMAL.

		PHAGOCYTOSIS		
Serum + m / 8 NaCl Solution, 5 Per Cent Suspension Washed Corpuscles, Sus. Washed Leucocytes of Dog, Equal Parts		Immune Serum		Normal Serum
		O. T. C. C.	O. OI 25. C. C.	O. T. C. C.
Goat corpuscles + dog leucocytes	++ +	++	o
Sheep	++ +	+	o
Dog	++ +		o
Guinea-pig	++ +	o	o
Rabbit	++ +	o	o
Human	++ +	o	o
Chicken	++ +	o	±
Pigeon	++ +	o	o
Beef	++ +	o	o
Goat	o	o	o
Guinea-pig leucocytes	++ +	+	o
Sheep	++ +	o	o
Dog	o	o	o
Guinea-pig	o	o	o
Rabbit	o	o	o
Human	o	o	o
Chicken	o	o	o
Pigeon	o	o	o
Beef	o	o	o

+ = Phagocytosis good.
± = " trace.

Here it is shown also that normal rabbit serum has but little hemopsonic effect. The hemopsonic power of normal rabbit as well as of other sera appears to vary considerably, however, and the susceptibility of erythrocytes of various animals to opsonification is also a variable quality. At all events the amount of hemopsonin in normal serum would seem to be much smaller than that of bacteriopsonin.¹ Barratt found that normal sera may contain erythrocytic opsonins in small amounts.

The results obtained in similar experiments with other immune hemopsonins are shown in Table 2. It is of course possible that

TABLE 2.
RANGE OF OPSONIC POWER OF IMMUNE HEMOPONINS.

Serum + m/8 NaCl Sol. 5% Suspension Washed Corpuscles, Suspension Washed Leucocytes (Dog) Equal Parts	PHAGOCYTOSIS							
	Goat Serum				Guinea-Pig Serum		Rabbit Serum	
	Immune to Sheep Blood			Normal	Immune to Rabbit Blood	Normal	Immune to Beef Blood	Normal
	0.1C.C.	0.025C.C.	0.006C.C.	0.1C.C.	0.1C.C.	0.1C.C.	0.1C.C. and 0.025C.C.	0.1C.C.
Goat corpuscles.....	++	+	o	o	o	o	++	o
Sheep ".....	+++	+++	+	o	o	o	o	o
Dog ".....	o	o	o	o	o	o	o	o
Rabbit ".....	Tr*	o	o	o	++	o	o	o
Guinea-pig ".....	Tr*	o	o	o	o	o	o	o
Human ".....	Tr*	o	o	o	o	o	o	o
Beef ".....	+	o	o	o	o	o	+++	o

*Tr=trace.

larger quantities of serum or more potent serum might be found to contain opsonins for other corpuscles than those that are opsonized by these sera. Variable susceptibility of the erythrocytes as well as variation in the phagocytic activity of the leucocytes employed may also cause some variation in the results, but in nearly all the sera tested the opsonic effect on dilution becomes limited quite strictly to the corpuscles used for immunization.

The question now arises whether the immune sera that opsonize several kinds of corpuscles do this by virtue of a single opsonic sub-

¹ In connection with the question of the specificity of hemopsonins it is of interest to note that Bulloch and Western (*Proc. Roy. Soc., B*, 1906, 77, p. 531) have found that opsonins for staphylococci and tubercle bacilli in the serum of normal and vaccinated human beings are distinct substances.

stance or several such. Certain absorption experiments that I have made show that while the corpuscles used for immunization remove all the opsonins in the serum, other corpuscles that are opsonized by the serum may absorb only those opsonins that appear to be peculiar to themselves. Table 3 shows the results of experiments

TABLE 3.

SEPARATION OF SPECIFIC AND COMMON HEMOPSONINS IN IMMUNE SERA.

ABSORPTION OF OPSONIN IN SERUM OF GOAT IMMUNE TO SHEEP BLOOD BY (1) GOAT CORPUSCLES AND
(2) SHEEP CORPUSCLES.

1.

Immune serum 0.05 + 10 per cent suspension washed goat corpuscles 3 c.c. After one hour at 37° C.
centrifugate.

Supernatant fluid 1.5 (0.025 serum) + 5 per cent susp. sheep
corpuscles 0.2 + susp. dog leucocytes 0.2 = Phagocytosis

Supernatant fluid 1.5 (0.025 serum) + 5 per cent susp. sheep
corpuscles 0.2 + susp. goat leucocytes 0.2 = No “

Immune serum 0.025 + 5 per cent susp. sheep corpuscles 0.2
+ susp. goat leucocytes 0.2 = Phagocytosis

2.

Immune serum 0.05 + 10 per cent suspension washed sheep corpuscles 3.3. After one hour centrifugate

Supernatant fluid 1.5 (0.025 immune serum) + 5 per cent
washed corpuscles goat 0.2 + dog leucocytes 0.2 = No phagocytosis

Supernatant fluid 1.5 (0.025 immune serum) + 5 per cent
washed sheep corpuscles 0.2 + dog leucocytes 0.2 = Trace “

Immune serum 0.025 + 5 per cent washed sheep corpuscles
0.2 + dog leucocytes 0.2 = Phagocytosis

ABSORPTION OF OPSONIN IN SERUM OF RABBITS IMMUNE TO GOAT BLOOD BY (1) SHEEP CORPUSCLES AND
(2) GOAT CORPUSCLES.

1.

Immune serum 0.05 + 5 per cent suspension of washed sheep corpuscles 2.4 c.c. Centrifugate after one
hour at 37° C.

Supernatant fluid 0.6 (0.0125 of serum) + 5 per cent susp. goat
corpuscles 0.2 + suspension dog leucocytes 0.2 = Good phagocytosis

Supernatant fluid 0.6 (0.0125 of serum) + 5 per cent susp.
sheep corpuscles 0.2 + suspension dog leucocytes 0.2 = No “

Immune serum 0.0125 in NaCl sol. 0.6 + 5 per cent susp.
sheep corpuscles 0.2 + suspension dog leucocytes 0.2 = Good “

2.

Immune serum 0.05 + 5 per cent suspension of washed goat corpuscles 2.4 c.c. Centrifugate after one
hour at 37° C.

Supernatant fluid 0.6 (0.0125 of serum) + 5 per cent susp.
sheep corpuscles 0.2 + suspension dog leucocytes 0.2 = No phagocytosis

Supernatant fluid 0.6 (0.0125 of serum) + 5 per cent susp.
goat corpuscles 0.2 + suspension dog leucocytes 0.2 = No “

Immune serum 0.0125 in NaCl sol. 0.6 + 5 per cent susp.
goat corpuscles 0.2 + suspension dog leucocytes 0.2 = Marked “

of this kind with the serum of goats immune to sheep blood, and with the serum of rabbits immune to goat blood. Similar results have

been obtained with the serum of rabbits immune to beef blood. These experiments warrant the conclusion that immune hemopsonic serum may contain specific and non-specific or common opsonins in every way analogous to the specific and common or group agglutinins,¹ and precipitins that develop in animals immunized with the proper antigens. Whether blood relationship can be brought out by means of immune hemopsonins after the manner that Nuttall has shown serum-precipitins do would seem an interesting problem.

PHAGOCYTOSIS OF HUMAN ERYTHROCYTES BY HUMAN LEUCOCYTES.

The often extensive phagocytosis of red corpuscles and of other cells, especially in the spleen, the marrow, the lymph and hemolymph nodes in various infectious, toxic, and anemic processes, suggested that a study of phagocytosis of human erythrocytes by human leucocytes *in vitro* from the point of view of opsonification might be of interest. For this purpose small quantities of citrated human blood from various sources are washed three or four times in liberal quantities of NaCl solution by means of centrifugalization; in order to secure as many leucocytes as possible the superficial layers of the sedimented blood corpuscles, the "blood cream," are made into a thick suspension in salt solution, and 0.1 or 0.2 c.c. of this suspension is mixed with an equal quantity of the serum, the opsonic effect of which is to be determined; this mixture is then placed at 37° C. for 40 minutes when smears are made and stained.

A brief summary of the essential facts obtained is displayed in Table 4. In no case so far has phagocytosis been observed in mixtures of washed fresh blood cream and salt solution only. Serum is essential for phagocytosis here also. Erythrocytes may take up the active substance—hemopsonin—from the serum and in so doing they become susceptible to phagocytosis by washed leucocytes after every trace of serum has been removed by repeated washing. If sufficient erythrocytes of the proper kind are added, all the opsonin may be removed from the serum.

As shown in Table 4 the sera of patients with various infections and occasionally also the sera of normal persons may be agglutinating

¹ See William Hallock Park, *Jour. Infect. Dis.*, 1906, Suppl. No. 2. p. 1; also Park and Collins, *Jour. Med. Res.*, 1901, 7, N. S., p. 491.

or opsonic, most commonly both, for human erythrocytes, especially, it would seem, those from patients with certain diseases, e. g., pneumonia. Phagocytosis without agglutination may occur, but that is rare; agglutination without phagocytosis is more common; agglutination and phagocytosis most common if the serum has any action whatsoever upon the red corpuscles used. This phagocytic and agglutinative property of certain sera, especially, so far as my experience goes, of some typhoid patients appears to be lost very slowly on standing (ice-chest). In other sera it may be lost more quickly.

TABLE 4.

OPSONINS AND AGGLUTININS FOR HUMAN CORPUSCLES IN HUMAN SERA.

WASHED BLOOD CREAM + SERUM EQUAL PARTS	PHAGOCYTOSIS AND AGGLUTINATION BY VARIOUS SERA													
	Typhoid F. 1		Scarlet F.		Typhoid F. 2		Erysipelas		Pneumonia		Normal		Normal	
	Aggl.	Phg.	Aggl.	Phg.	Aggl.	Phg.	Aggl.	Phg.	Aggl.	Phg.	Aggl.	Phg.	Aggl.	Phg.
Pneumonic blood cream	+++	+++	+++	++	+	++	++	++	o	+++				
Typhoid blood cream...	+++	+	o	o			++	±						
Scarlet F. blood cream...	+++	+++	+++	+	++	+								
Normal blood cream...			+	o	++	+	+	o	o	o				
*Erysipelas blood cream					++	+	+++	*+						
*Pneumonic blood cream					+++	++	++	+	+++	*+				
Rheumatic blood cream			o	o			o	o			o	o		
Cellulitis blood cream...			o	o			o	o			o	o		
Normal blood cream...			+	o	+	+	+	o	o	o	o	o		
Normal blood cream...			++	+							++	+		
Pneumonic blood cream			++	++									++	++
Normal blood cream...			o	o									o	o

+++ = phagocytosis well marked. ++ = phagocytosis marked. + = phagocytosis present.

*In these cases it concerns serum and blood cream from the same person (autophagocytosis or autoagglutination).

As indicated the same serum may be opsonic and agglutinating for certain corpuscles but not for others, showing clearly that the condition of the corpuscles may vary greatly. Whether this variation (which I have noticed even in the corpuscles of the same person at different times) may depend on the lack of suitable receptors or upon resistance to the functional group of the agglutinin or opsonin must be determined by further study. As regards the opsonification.

I have found that the same corpuscles may be sensitized for phagocytosis by leucocytes of one blood but not by those of another, indicating that the state of the leucocyte also may vary. It is too early to make any definite statements as to the erythrophagocytic power of leucocytes and to the susceptibility to opsonification of erythrocytes in different diseases, but I have been struck particularly with the readiness with which phagocytosis (and agglutination) is obtainable in pneumonic blood cream and with the opsonic power of certain typhoid sera. On the whole my observations as to hemagglutinins in human serum correspond with those previously recorded by Eisenberg,¹ Herter and others.²

So far I have not encountered any instance of lysis of human red cells by human serum.

As shown in the table the serum of an individual may be agglutinative for the corpuscles of that individual and also subject them to phagocytosis by their fellow leucocytes. In other words, serum may be auto-agglutinative and auto-opsonic. My colleagues, Dr. Rosenow and Dr. Davis, on several occasions have noted autophagocytosis in defibrinated blood of patients with pneumonia and with acute (meningococcus) meningitis. Wright³ observed phagocytosis of red cells in two cases of pneumococcus infection (cellulitis, cystitis). He suggests that this may be due to toxic damage to the red cells which then are taken up without the intervention of opsonin. The extent to which this explanation will hold remains to be seen. We know that phagocytosis of some objects—certain bacteria, coal particles, carmin granules—may occur without the presence of serum and consequently presumptively without opsonin. Hence it is possible that altered red cells may be taken up without opsonification and the exact mechanism of erythrophagocytosis under different conditions merits careful consideration. There can be no question, however, that in certain diseases, notably typhoid fever, the serum acquires the power to promote phagocytosis by its opsonic action upon the red cells. I have not been able to demonstrate the presence of hemopsonins in cultures of typhoid bacilli, and it would seem that the hemopsonin in the serum of typhoid patients develops as the result of reactions

¹ *Wien kl. Wchnschr.*, 1901, 14, p. 1021.

³ *Brit. Med. Jour.*, 1906, Jan. 20, p. 143.

² *Medical Record*, 1902, 61, p. 117.

in the infected body. Mallory¹ suggested that the phagocytosis of erythrocytes and other cells in typhoid fever is due to stimulation of phagocytes by bacterial toxins. Naturally the demonstration of hemopsonins will lead to special stress being placed on the importance of opsonification in infectious erythrophagocytosis.

As already mentioned Savtchenko, Levaditi, and Gruber showed that the injection of the serum of rabbits immunized with guinea-pig blood into the peritoneal cavity of guinea-pigs is followed by marked erythrophagocytosis in the peritoneum as well as in the blood-making organs, especially the spleen and also in the blood (Levaditi). Portis² observed that in dogs injected with the serum of goats, immunized with dog thyroid, there occurred an extensive phagocytosis of red corpuscles by hyaline and endothelial cells.³ The occurrence of phagocytosis of red cells *in vivo* under the influence of sera containing specific hemopsonins consequently appears well established. On the whole it seems reasonable that the development of hemopsonins and consequent phagocytosis, in some degree at least, will serve to explain the anemia of infections in which erythrophagocytosis occurs.

On injection of immune serum Stschastnyi⁴ noted, in the first place, phagocytosis of red cells, and later, so he believes, the gradual transformation of the phagocytic leucocytes into typical eosinophils, the granules of which he derives from hemoglobin. Whether, in case this view of the nature of eosinophils becomes general, hemopsonins play any part in their formation under normal and pathological conditions, e. g., after infections, must be left to future study. Paroxysmal hemoglobinuria as well as other destructive blood diseases invite investigation from the point of view of hemopsonins. Eason⁵ describes phagocytosis of normal red corpuscles (human) by

¹ *Jour. Exp. Med.*, 1898, 3, p. 611, and 1900, 5, p. 1.

² *Jour. Infect. Dis.*, 1904, 1, p. 127.

³ In connection with this observation it may be worth while to record the results of a small series of experiments with cytotoxic sera. At my request Dr. S. P. Beebe of New York kindly sent me sera of Belgian hares injected with nucleoproteids of the adrenal and the kidney of the dog and of the thyroid of a fatal case of exophthalmic goiter. Of these sera the anti-adrenal had no opsonic effect upon beef, sheep, goat, dog, rabbit, or human corpuscles, using dog leucocytes as phagocytes. The anti-renal had only slight effect on goat and sheep corpuscles. The anti-thyroid contained traces of opsonin for goat, sheep, dog, rabbit, and guinea-pig corpuscles and opsonin in large quantities for human erythrocytes.

⁴ *Ziegler's Beiträge*, 1905, 38, p. 456.

⁵ *Edin. Med. Jour.*, 1906, 19, N. S., p. 43.

normal leucocytes in the presence of the serum of hemoglobinuric patients. And we know that in pernicious anemia there is phagocytosis of red cells.

SUMMARY.

Normal serum may contain opsonins for heterologous, and in some instances also (human serum) for homologous, erythrocytes.

Repeated injections with alien blood commonly give rise to the accumulation of hemopsonins in the blood.

Immune hemopsonic serum may contain common or non-specific hemopsonins as well as specific hemopsonins directed particularly against the corpuscles of the blood employed for the injection.

Immune hemopsonins possess a high degree of resistance to heat and other influences.

Hemopsonins render red corpuscles susceptible to phagocytosis by various leucocytes, including the homologous, but the phagocytic activity of different leucocytes toward opsonized erythrocytes may vary.

Human serum may contain agglutinin and opsonin for human erythrocytes. This appears to be the case especially in typhoid fever. The corpuscles of various individuals vary in their susceptibility to agglutination and phagocytosis by human leucocytes and the phagocytic power of leucocytes may vary. Auto-agglutinins and auto-opsonins appear to occur.

The demonstration of opsonins for human corpuscles in human serum may help us to a better understanding of the phagocytosis of erythrocytes in infectious and other processes.

OBSERVATIONS ON THE OPSONIC INDEX AND THE ANTIPNEUMOCOCCAL POWER OF THE BLOOD IN PNEUMONIA.*

HERMAN E. WOLF.

(From the Pathological Laboratory of Rush Medical College, Chicago.)

It has been shown by Wright and Douglas and others that phagocytosis of various bacteria is dependent upon the presence in the blood and other fluids of certain substances called opsonins; moreover, that these substances exert their influence upon the bacteria and not directly upon the leucocytes. It has been demonstrated also that opsonins are increased during the course of acute infections, especially as recovery takes place, and that they may be artificially increased by the injection of killed bacteria. Thus Wright and Douglas¹ have shown that there may be a deficiency of the proper opsonins in chronic staphylococcus infections and in tuberculosis, and that the opsonins are increased by injection of corresponding bacterial vaccines. Mennes² previously described an increase in the substance that promotes phagocytosis of pneumococci in the serum of animals immunized to pneumococci. He took the view then prevalent that the substance in question acted wholly by stimulating the leucocytes to phagocytic action. Hektoen³ obtained increase in the streptococcic opsonic index in man by the injection of dead streptococci, while Neufeld and Rimpau⁴ found that sera of animals immunized to streptococci and pneumococci prepare virulent strains of these bacteria for phagocytosis, while normal sera do not.

It was thought, therefore, that it would be of interest to determine the opsonic power of the blood during the course of pneumonia in its various phases. Corresponding dilutions of normal and pneumonic serum were added to fixed quantities of washed blood (leuco-

* Received for publication June 4, 1906. This work was conducted under the Alumni Fellowship of Rush Medical College, Chicago.

¹ References to the work of Wright and his colleagues are given by Wright, *Lancet*, 1905, 2, p. 1598.

² *Zeitschr. f. Hyg.*, 1897, 25, p. 413.

³ *Jour. Am. Med. Assoc.*, 1906, 46, p. 1407.

⁴ *Deutsch. med. Wchnschr.*, 1904, 32, p. 1459.

cytes) and pneumococcal suspension with the view of eliciting minute variations in the resulting phagocytosis. The quantities of 0.2, 0.1, 0.05, 0.025, 0.0125 c.c. of serum were tried, the quantity in each instance being made up to 0.2 c.c. and each tube contained in addition 0.2 c.c. of washed normal blood (leucocytes) and 0.2 c.c. of pneumococcus N suspended in normal salt solution. Pneumococcus N was isolated from the blood of a pneumonia patient three months before, and is quite readily taken up by the leucocytes under these circumstances. The mixtures were incubated at 37° C., and at the end of 20 minutes smears were made and stained, the number of organisms counted in at least 20 leucocytes in each case, and an average obtained. It was found that 0.025 c.c. gave the most satisfactory results, and the pneumococco-opsonic indexes in the charts are based on results so obtained.

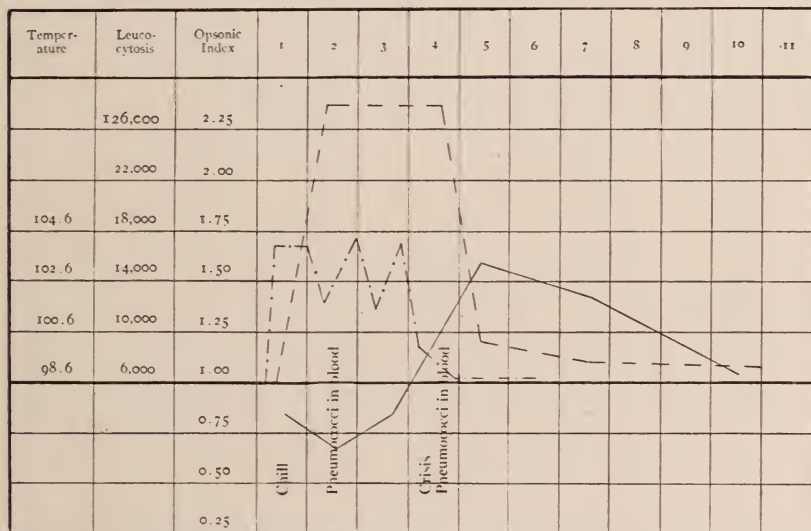
Eleven cases were studied by this method, usually with daily determinations before crisis in typical cases, and less frequently thereafter. This series includes two cases of pneumococcal empyema and two cases of migratory pneumonia, two fatal cases of pneumonia, while the remaining five were cases of typical lobar pneumonia terminating in crisis. Besides the determination of the opsonic index, leucocyte counts and blood cultures were made, the results of which are indicated also in the charts.

All the typical cases of pneumonia were found to have certain characteristics in common, and Chart 1 illustrates the type of variation in the pneumococco-opsonic index in typical pneumonia with definite crisis. It is noteworthy that the opsonic index persists below normal until within a few hours before crisis (the negative phase of Wright), then it rises above normal and reaches its height within 24 hours after crisis when decline toward normal takes place. The leucocyte curve, on the other hand, rises rapidly after the chill and begins to decline as the opsonic index rises remaining above normal for a few days after crisis.

While the opsonic index may be regarded as an index of the protective powers of the blood we must not leave out of sight the rôle of the polymorphonuclear leucocytes which are equally necessary for phagocytosis. As the leucocytes are enormously increased in pneumonia the antipneumococcal power of the blood may be much

greater than is apparent from the study of the opsonic curve only. If each leucocyte in normal serum takes up 4 pneumococci and there are 4,200 such polynuclear leucocytes per c.mm. of normal blood, we may consider that $4,200 \times 4 = 16,800$ organisms might be taken up in each c. mm. of blood. If 3 pneumococci are taken up by each pneumonic leucocyte in pneumonic serum during the negative phase and there are 18,700 leucocytes per c.mm., the total number of organisms taken up will be $3 \times 18,700 = 56,100$, which would represent the entire antipneumococcic power of 1 c.mm. of pneumonic

CHART 1.
PNEUMOCOCCO-OPSONIC INDEX IN TYPICAL PNEUMONIA ENDING IN CRISIS.



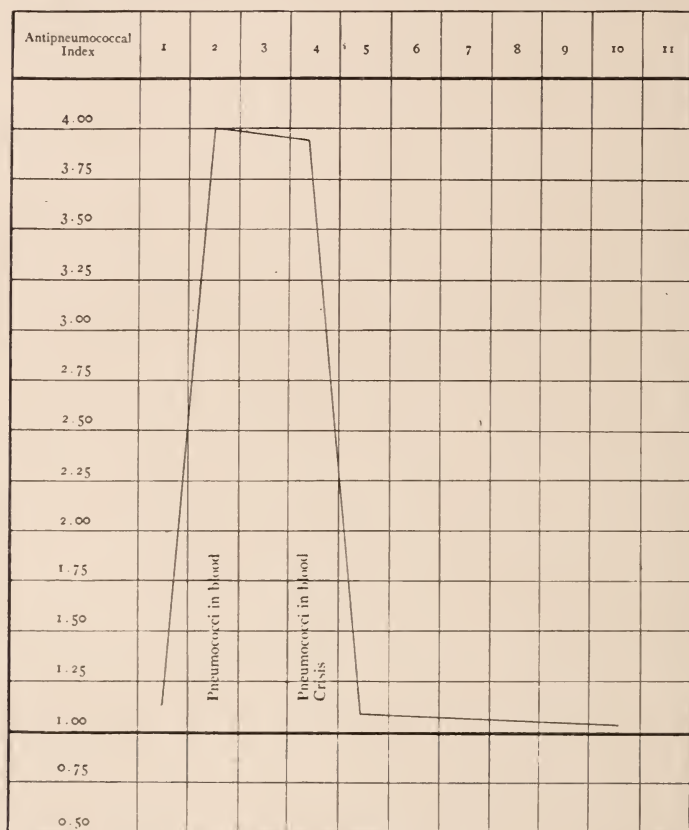
blood. It is therefore readily seen that although the pneumococco-opsonic index of pneumonic blood may be below normal early in pneumonia, the antipneumococcal power of the same may be $3\frac{1}{3}$ times the normal, assuming of course that the opsonin present is sufficient to opsonize the quota of pneumococci necessary. It is noteworthy that Rosenow's¹ observations upon the rôle of polymorphonuclear leucocytes in the pneumococcidal action of human blood *in vitro* go to support the view here taken.

¹ *Jour. Infect. Dis.*, 1906, 3, p. 683.

This possible relative antipneumococcal value of the blood of a typical case of pneumonia (Chart 1) I have plotted on Chart 2, from which we see that although the pneumococco-opsonic index (Chart 1) is below normal during the first two days, the actual anti-pneumococcal power would be far above normal. This relation appears to

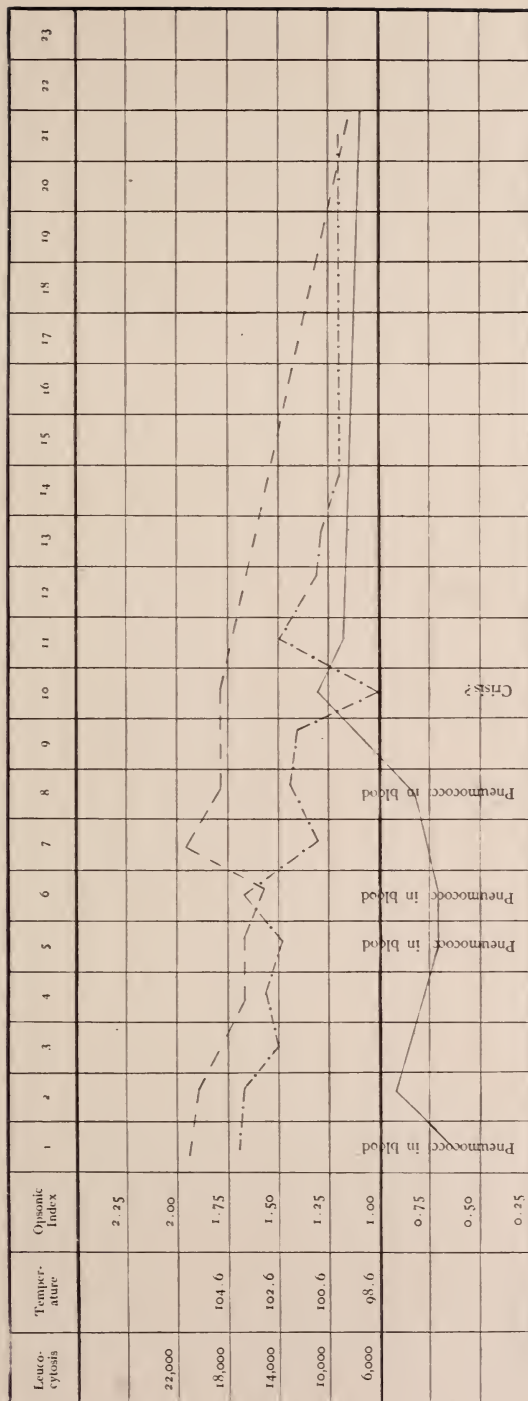
CHART 2.

ANTIPNEUMOCOCCAL POWER OF THE BLOOD IN A TYPICAL CASE OF PNEUMONIA.



continue until the opsonins are at their height, i. e., shortly after crisis, when the leucocytes have so decreased that the antipneumococcal power of the blood would be decidedly lowered though still above normal. The highest point reached thus appears to be shortly before crisis is complete.

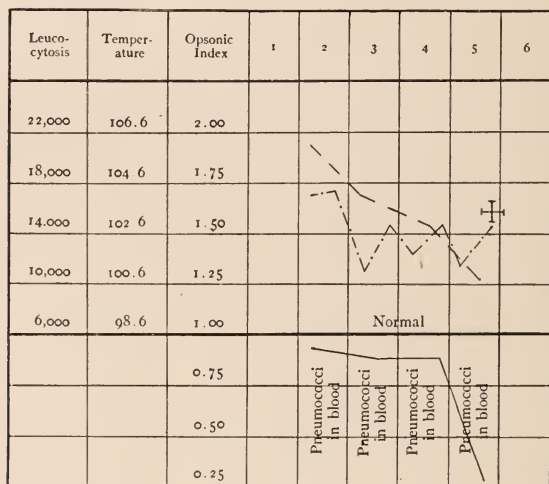
CHART 3.
OPSONIN, LEUCOCYTE, AND TEMPERATURE CURVES IN A CASE OF MIGRATORY PNEUMONIA.



— Opsonin.
 --- Leucocytes.
 - . - . - . Temperature.

Two cases of migratory pneumonia were studied and the conditions are illustrated in Chart 3. This particular case was first seen on its fourth day after the chill, when the opsonic index was below normal, rising somewhat the next day. Then extension of the process in the lung took place, three lobes becoming successively involved, the pneumococco-opsonic index declining. On the twelfth day of the attack the opsonins rose above normal and reached their height on the next day when the temperature became normal for the first time. There was delayed resolution and slight fever until

CHART 4.
PNEUMOCOCCO-OPSONIC INDEX IN FATAL PNEUMONIA.



————— Opsonin.
 - - - - - Leucocytes.
 - . - . - Temperature.

the 25th day, when resolution become complete. During this time the opsonic index remained above normal.

It is interesting that in the fatal cases examined the opsonic index continued to fall until death. In the case represented on Chart 4 considerable leucocytosis was present and the total anti-pneumococcal power might be conceived as still considerably above normal. It is of interest to note that the number of leucocytes was falling steadily. In other cases with a decided hypoleucocytosis and a low pneumococco-opsonic index the total antipneumococcal

power would certainly be low and perhaps explain, to some extent, the fatal results, though many more cases must be examined before definite conclusions may be drawn.

Opsonins in metapneumonic pleuritic and arthritic exudates.—The pus from four cases of postpneumonic empyema and one case of pneumococcal arthritis was obtained and centrifugated. The supernatant fluid so obtained was found to be devoid of opsonins. Varying quantities of this fluid were added to washed human corpuscles (leucocytes), normal and suspensions of organisms in order to test whether it possessed antileucocytic and antiphagocytic power. Table 1 shows that these fluids have no power to suspend phagocytosis.

TABLE 1.
INFLUENCE OF PNEUMOCOCCAL EXUDATES ON PHAGOCYTOSIS.

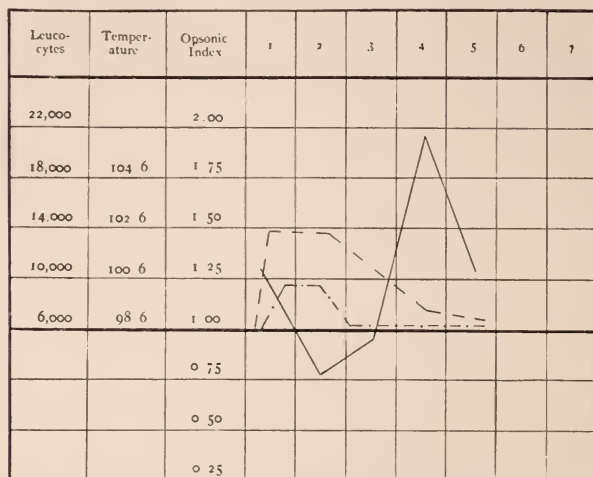
MIXTURES	PHAGOCYTOSIS	
	20 Min.	1 Hr.
Pleuritic exudate 0.4 c.c. + washed corpuscles 0.2 c.c. + serum 0.2 c.c. + suspension 0.2 c.c.	4	10
Pleuritic exudate 0.2 c.c. + washed corpuscles 0.2 c.c. + serum 0.2 c.c. + suspension 0.2 c.c.	6	9.8
Normal salt solution 0.4 c.c. + washed corpuscles 0.2 c.c. + serum 0.2 c.c. + suspension 0.2 c.c.	4	11
Normal salt solution 0.2 c.c. + washed corpuscles 0.2 c.c. + serum 0.2 c.c. + suspension 0.2 c.c.	6	10.2
Pleuritic exudate 0.2 c.c. + washed corpuscles + suspension 0.2 c.c.	0	0

Production of immune opsonins by injection of pneumococcal vaccines.—It has already been mentioned that the opsonins in the blood may be increased by the injection of bacterial substances or vaccines (Wright). The following experiment may be given as a further illustration: A healthy man was injected under the skin of the arm with the 24-hour growth of 10 blood-agar slants of virulent pneumococcus S heated to 60° C. for one hour. This was followed in four hours by a leucocytosis of 13,800 persisting for 48 hours together with slight rise in temperature as shown in Chart 5. There were also some malaise and headache, while locally there was considerable pain, reddening, and infiltration which disappeared without softening in 48 hours. The general symptoms also subsided within 36 hours.

The train of events as seen from Chart 5 correspond well with the changes that occur in cases of pneumonia—at first there is a

CHART 5.

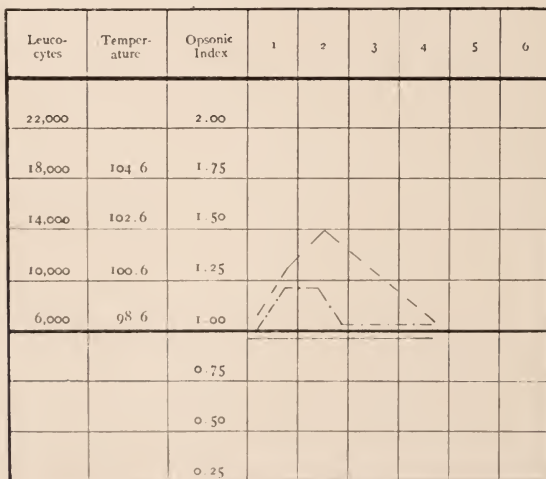
INFLUENCE OF DEAD VIRULENT PNEUMOCOCCI UPON THE OPSONIC INDEX IN A NORMAL INDIVIDUAL.



————— Opsonin.
 - - - - - Leucocytes.
 - . - . - . - Temperature.

CHART 6.

INFLUENCE OF DEAD AVIRULENT PNEUMOCOCCI UPON THE OPSONIC INDEX OF A NORMAL INDIVIDUAL.



————— Opsonin.
 - - - - - Leucocytes.
 - . - . - . - Temperature.

rapid decrease in opsonins, followed by rapid rise with a decline to normal. It is to be noted that the leucocytes here also begin to drop as the opsonins rise.

As it had been noted that dead avirulent pneumococci produce as marked a temperature and leucocyte reaction as do dead virulent cultures, another experiment was made in which dead avirulent pneumococci were substituted for the virulent ones. The result is shown in Chart 6. The temperature and leucocyte reactions are comparable to those in the experiment with virulent pneumococci but no appreciable difference was exerted upon the pneumococco-opsonic index. Now we know that virulent pneumococci, dead or alive, *in vitro* are not susceptible to phagocytosis in normal or pneumonic blood. It is therefore possible that the property of virulence is concerned in stimulating the production of immune opsonins observed in Chart 5 but lacking in Chart 6.

Effect of pneumococcal vaccines in pneumonia.—It has been found that opsonins can be increased by injections of the proper bacterial vaccines and that this increase is in many cases associated with a favorable influence upon the infection. Whether this salutary effect can be explained wholly by the increase in opsonins, as some are inclined to think, is perhaps questionable, yet the opsonic index certainly may be taken as a sort of measure of the resistance as shown from the foregoing observations.

In all, 14 cases of lobar pneumonia were treated by the injections of pneumococcal vaccines, of which 11 recovered and 3 ended fatally, as indicated in Table 2. In all cases with a favorable termination there was a very slight rise of temperature from four to six hours after the injection together with an increased leucocytosis. Crisis also occurred from 18 to 36 hours after the injection in all but one case, where a second injection was given and crisis took place 18 hours after the last injection. The opsonins, below normal at the first determination, rose characteristically just before crisis, though no exaggeration of the negative phase occurred immediately after the injections. In other words, the evolution of the course was precisely that of a case untreated, with the exception that crisis seemed to occur early. In the 11 cases that recovered, five cases had the crisis on the third day, three on the fourth, one on the fifth, and two

on the sixth. These cases were chosen at random from cases with a mortality of about 40 per cent for that month; hence the number of recoveries among the injected would seem to be high, and certainly crisis occurred unusually early.

TABLE 2.
EFFECT OF INJECTIONS OF PNEUMOCOCCAL VACCINES IN PNEUMONIA.

PATIENT	DAY OF DISEASE	LEUCOCYTES		TEMPERATURE		CRISIS OR DEATH			INVOLVEMENT
		Before Injection	Four hrs. after Injection	At Injection	Four hrs. after Injection	Result	Day of Disease	After Injection	
F. G.	3d	19,200	30,000	103.4	103.8	Crisis	4th	34 Hrs.	L. l. lobe
A. B.	3d	17,200	24,000	102.2	102.6	"	4th	26 "	R. l. lobe
A. E. B.	2d	101.2	101.8	"	3d	40 "	R. l. and r. m. lobes
J. C.	2d	101.4	101.8	"	3d	24 "	Rt. side complete
M. D.	3d	27,100	28,000	102.2	102.2	"	4th	22 "	R. l. lobe
Sch.	4th	37,000	38,000	103.0	103.4	"	5th	24 "	L. l. lobe
P. K.	6th	25,500	26,500	102.8	103.0	"	6th	18 "	R. l. and r. m. lobes
A. S.	3d	12,000	18,000	102.0	102.4	"	3d	16 "	L. l. lobe
T. M.	2d, 3d	22,600	29,000	103.0	103.4	"	6th	30 "	Complete rt. side
A. B.	2d	19,200	26,000	102.6	103.2	"	3d	36 "	L. l. lobe
A. H.	2d	14,000	20,000	103.0	103.4	"	3d	26 "	L. l. lobe
L. W.	2d	17,000	14,000	102.2	102.0	Death	6th	96 "	Rt. upper and mid. and l. l. lobes
A. H.	4th	14,000	12,250	101.2	101.0	"	5th	18 "	Complete l. lung
C. H.	3d	4,000	3,100	103.8	103.6	"	5th	42 "	Rt. u. and m. lobes

Of the three cases ending fatally two were senile pneumonias, while the other showed a marked hypoleucocytosis. In these cases after injection no marked temperature or leucocyte reaction occurred, nor was it followed by any increase or decrease in opsonins. This condition of affairs might be construed to mean that the sources of vital resistances of the body had been so thoroughly exhausted as to permit of no further stimulation.

SUMMARY AND CONCLUSIONS.

1. In pneumonia the pneumococco-opsonic index is first decreased, but rises in favorable cases, reaching its height soon after crisis, while in fatal cases it remains persistently low.

2. The estimated total antipneumococcal index (estimated from the leucocytic index and the opsonic index) is early increased and remains high until crisis is complete in cases with favorable termination.

3. Pneumococcal exudates contain little or no pneumococco-opsonin nor do they exert any antiphagocytic action.

4. Dead virulent pneumococci produce a similar evolution in the antipneumococcal index in normal persons to that which occurs in pneumonia.

5. Dead avirulent pneumococci have no effect on the pneumococco-opsonins.

6. Treating patients with lobar pneumonia with pneumococcal vaccines seems to exert a favorable influence upon the course of the disease, as indicated by an apparent decrease in mortality and by early crisis, but a great deal of further work is necessary before any conclusions of final value may be drawn.

I herewith wish to express my thanks to the Rush Alumni Association, for making the work possible; to Dr. Hektoen, under whose direction it was done; and to the house staff of the Cook County Hospital for the many courtesies shown.

CONCERNING THE PRODUCTION OF CYTOTOXIC SERA BY THE INJECTION OF NUCLEOPROTEIDS.*†

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THIS investigation was incited by the publication of Beebe's studies¹ which describe the production of specific cytotoxic sera for the liver, kidney and pancreas as the result of treating animals with the respective nucleoproteids of these organs. As one of us² had previously made a somewhat extensive study of cytotoxic sera prepared by the injection of various somatic cells, and had reached conclusions which did not justify the views then current concerning the specificity of these bodies, it seemed advisable, in view of Beebe's very definite results, to further pursue these studies.

THE DEVELOPMENT OF OUR KNOWLEDGE OF THE CYTOTOXINS.

It may not be out of place to interpolate here a few words concerning the very definite change in opinion which has occurred during the past few years concerning the specificity of the cytotoxins. Shortly after the early publications of Ehrlich and his associates concerning the production of immune hemolysin, a vast number of publications describing specific sera for practically all cells of the body appeared in rapid succession. In most of these studies an attempt was made to demonstrate a specificity according to morphological rather than receptorial affinity in disregard of the very clear and definite statement of Ehrlich and Morgenroth. Those who denied a morphological specificity based their opinion on the observation that by the injection of cells, as those of the testicle, apparently free from blood, there resulted not only an antiserum for these cells, but also an hemolysin. This was explained by those who believed in morphological specificity by assuming that a number of red blood corpuscles, sufficient to produce a hemolysin, had been injected with the semen. Later, however, the production by a large number of investigators of hemolysin by the injection of fluids entirely free from red corpuscles, as serum and urine, demonstrated the production of antibodies from free receptors and firmly established Morgenroth's contention that specificity is a matter, not of cells, but of receptors. Despite these very conclusive demonstrations later investigators, who attempted to pro-

* Received for publication September 13, 1906.

† Conducted under a grant from the Rockefeller Institute for Medical Research. Read before the Section in Pathology of the British Medical Association, Toronto, August 21, 1906.

duce antisera for the cells of various organs, continued to use emulsions of unwashed organs in utter disregard of the presence of free receptors in the organ juices and also without consideration of the antibodies certain to be produced by the red cells normally present. That sera so produced possessed very definite hemolytic and hemagglutinative properties capable of producing lesions (hemoglobinuria, thrombosis, liver necroses) which might erroneously be ascribed to the supposedly specific body was first suggested by Pearce³ in a study of nephrotoxins and later² definitely demonstrated by a study of the action of sera prepared from the liver, kidney, pancreas, and adrenals completely freed from blood by prolonged washing through the blood vessels. Further, the study of sera so prepared and also of sera resulting from the injection of blood serum, bile, and urine, led to the conclusion that "the cells of various organs of the body have, while differing in morphology and function, certain receptor characteristics in common, and that one type of cell may therefore produce antibodies affecting several cells of differing morphology but with like receptor groups." Specificity in the morphological sense could not be demonstrated.

Other investigations with sera prepared from washed organs, especially those of Portis⁴ (antithyroid serum) and Woltmann⁵ also indicate the non-specificity of the cytotoxins.

Thus, while the study of the cytotoxins has aided and may still aid in the development of our knowledge of immunity and has incidentally offered several valuable methods of experimentally reproducing pathological conditions, it is evident that the sera produced by the injection of mixtures of organ cells have not a degree of specificity sufficient to warrant their further study in the hope of developing therapeutic methods.

IMMUNIZATION TO NUCLEOPROTEIDS.

In order to prevent the adventitious formation of those bodies resulting from impure methods of immunization and also in the hope of obtaining greater specificity, a few investigators have utilized the proteid constituents of the cell.

Such attempts to produce antisera for somatic cells are limited, so far as we are aware, to those made by Marrassini,⁶ Bierry⁷ and his associates, and by Beebe. Of these studies the most comprehensive and most recent is that of Beebe.

Marrassini, working with the rabbit and guinea-pig, studied the effect of serum prepared by injecting rabbit's liver nucleoproteid. The changes resulting were, in general, degenerative in nature and similar to those caused by direct injection of the nucleoproteid itself, but usually more severe. The serum affected not only the organ for which it would be assumed it had an affinity, but also, to a greater extent, the kidney. Marrassini concludes that the action of a cytotoxin is of the same nature as that of other poisons of general action, such as potassium chromate, vinylamin, and pyrogallallic acid, which are predisposed to affect more especially the excretory organs.

The experiments of Bierry and his associates are few in number and reported but meagerly. In the first communication Bierry describes the action of a serum prepared by injecting rabbits with the nucleoproteids of the dog's kidney. The injection of 20 to 24 c.c. of the serum into the peritoneal cavity of a dog weighing 12 to 15 kilos caused, after three or four days, an albuminuria which reached its maximum on the 10th to the 15th day. The number of experiments is not stated, but

mention is made of one dog in which an albuminuria persisted for one and a half months.

Later in association with Pettit, Bierry made a comparison between the action of liver and kidney nucleoproteid sera. Each was found to cause albuminuria and degenerative changes in both liver and kidney, but the kidney serum produced a more severe albuminuria and more marked histological changes in the kidney than did the liver serum. The lesions of the kidney are described as interstitial hemorrhage, congestion of the glomeruli, and granular degeneration of the cells of the tubules with granular tube casts; and those of the liver as congestion and granular and vacuolar degeneration. The authors concluded that the action of such sera is more energetic upon the organ from which the nucleoproteid is derived, but that there is also a definite action on other organs.

In a third investigation Bierry, assisted by Mayer, made a special study of the urine of dogs receiving a liver nucleoproteid serum. They found biliary pigments, lactic acid, and homogentisic acid and demonstrated that, in some instances, the urine had a marked reducing power not due to the presence of dextrose. By various experiments they were able to produce a condition which they considered analogous to alimentary glycosuria and due to the changes produced in the liver by the serum. The histological changes in the liver were those of granular degeneration and fatty transformation. The kidney and pancreas were normal. Albuminuria occurred in two animals, but was slight and transitory. These results appear to contradict those given by Bierry and Pettit in the earlier communication.

Beebe prepared a serum by injecting thymus nucleohiston of the calf which gave precipitin tests for both nucleohiston and nucleoproteid of the thymus. The more important sera were those prepared by treating rabbits with the nucleoproteids of the liver, kidney, and pancreas of the dog. These all gave definite precipitin tests with their respective nucleoproteids and caused agglutination of cell mixtures, but had no lytic action upon the same. They were all markedly hemolytic and hemagglutinative *in vitro* in dilution up to one to five, but did not cause hemoglobinuria when injected into animals.

In testing their cytotoxic action *in vivo* intraperitoneal injections of 2 c.c. per kilo of body weight were usually employed. The kidney serum caused albuminuria and acute degeneration of the kidney without changes in other organs. Albuminuria appeared generally on the fourth or fifth day, increased in amount rapidly and was accompanied by the excretion of abundant hyaline and granular casts.

The liver serum caused granular and fatty changes and focal necroses of the liver with no changes in other organs or in the urine.

The urine of animals receiving pancreas serum caused what Beebe considers a partial reduction of Fehling's solution—"the formation of an abundant green precipitate, but not the typical red deposit of cuprous oxide." Albuminuria did not occur. Histological examination of the liver, kidney, and spleen revealed no changes; a study of the pancreas was impossible on account of imperfect fixation.

The results of these experiments (five with nephrotoxin, three with hepatotoxin and two with pancreas antiserum) provide, Beebe states, "strong indications that cytotoxic sera having a high degree of specificity can be prepared from the nucleoproteids of tissues," and that it is possible "to make a serum which will act primarily on one organ." Further comment on the work of Beebe will be made in the discussion of our own results.

METHODS.

As the amount of detail involved in work of this character is very great, it was considered advisable to limit our investigation to the study of two sera rather than to a superficial study of several. As physiological disturbances are the most satisfactory indications of the injurious action of a serum, antisera were prepared for the kidney and pancreas. The experience of practically all investigators that nephrotoxin is more nearly specific than other sera and the statements of Bierry and Mayer and that of Beebe concerning reducing substances in the urine also influenced this decision.

Rabbits, in groups of three, received in the peritoneal cavity five injections of the nucleoproteids of the dog's kidney and pancreas at intervals of five to seven days; after an interval of 10 days the animals were bled. The serum thus procured was injected intravenously into dogs which had been under observation for considerable periods of time and the urine of which had been carefully examined daily.

As a control of the supposedly specific nucleoproteinid sera, in order to determine if nucleoproteinid serum may not have a general toxic action on animals other than that from which the nucleoproteinid was prepared, rabbits were injected with the nucleoproteinid* of the pancreas of the cow and the effect of this serum was tried on the dog.

As a further control, and in order to determine also whether or not antisera for nucleic acids could be obtained, rabbits were injected with the nucleic acids of the dog's pancreas and kidney.

The preparation of the nucleoproteids.—In the preparation of the nucleoproteids we have employed one of the methods recommended by Levene. This seemed particularly advantageous for two reasons. In the first place the heating of the glands to the boiling-point effectually eliminates all possibility of future autolysis, while, on the other hand, the increased ease with which the solutions filter decreases the time of preparation. We are fully aware that the various nucleoproteids respond with different degrees of ease to extraction, but the point we had in view was not to obtain the maximum yield of material, but to endeavor to produce in the shortest time the purest product possible.

Emphasis must be laid upon the fact that only blood-free organs were employed. In this direction extreme care was exercised since the exclusion of all proteids of blood origin must be of prime importance where specific gland products stand in question. By inserting a cannula into the aorta below the renal arteries and laying a ligature above the coeliac axis, an irrigation of $\frac{n}{7}$ NaCl solution was established by means of which the blood was thoroughly removed from the organs. The blood-free organs immediately removed from the animal and cut into fine pieces were pressed with a mortar and pestle through a fine-mesh sieve and the finely comminuted material mixed with from two to four times its weight of water. The gland in suspension was then brought to the boiling-point in an Erlenmeyer flask of appropriate size. After allowing the mixture to cool it was quickly filtered through a double-folded filter. The first few cubic centimeters of filtrate, invariably turbid, were thrown on the filter again until a clear fluid resulted. The product of from one to two hours' filtration might be said roughly to correspond to the amount employed in each preparation.

* For this material we wish to thank Professor J. A. Mandel, of the University and Bellevue Hospital Medical School.

To this was then added acetic acid until complete precipitation resulted, the amount necessary varying according to the kind of nucleoproteid. Under ordinary circumstances the nucleoproteid separated out as a fine white flocculent precipitate; only once did it become necessary to add alcohol to bring about the precipitation of this character. This precipitate was washed with distilled water by sedimentation until the washings did not respond to the biuret reaction. This procedure in the absence of a centrifuge such as that described by Beebe was accomplished in four to five hours. In order to hasten the process, siphonage of the supernatant fluid took place before complete sedimentation had been accomplished even at the risk of loss of material. The material was then suspended in water and put into solution with the smallest possible amount of sodium carbonate; the resultant solution when filtered gave a clear filtrate which was reprecipitated with acetic acid, washed several times by decantation, and dehydrated with alcohol and ether. The products were white powders. That derived from the pancreas contained 1.74 per cent P; from the kidney 2.10 per cent P.

Just previous to each injection the required amount of the powder was weighed out and dissolved in an appropriate amount of sterilized 0.5 per cent sodium carbonate into which solvent it passed readily.

It seemed to us distinctly disadvantageous that the substance should be kept in solution for any length of time even by employing cold or antiseptics as precautions against autolysis or bacterial action. That changes of a certain character do take place under such circumstances is evidenced by the fact that Beebe noticed that a fine deposit settled out in his solutions after some weeks. In only one instance in our investigation was the injection made later than a few hours after the preparation of the solution. It would be hard to give absolute figures of the actual time consumed in the preparation of the various products. Excluding the period of cooling over night, when the gland stood in a condition free from the possibility of autolysis, the procedure occupied 15 hours most of which time, however, the material was suspended in water. This period, especially in view of the fact of the initial stoppage of autolysis, would not allow sufficient time for change of the character so guarded against by Beebe. In our opinion this precaution is undoubtedly necessary, but not of the extreme importance assumed by him. The freedom of the products from blood or protoplasmic proteid impurities would seem to be the goal for which to aim.

If, with Kossel, we assume that the present nucleoproteids or nucleins are in part laboratory products not originally present in the cell but formed in the process of the separation, the proteid moiety of the compound must be derived from protoplasmic substance as distinct from the chromatin, but not necessarily different from the cell proteids (globulins or albumins). If such is the case the question as to the specificity of nucleoproteids as such must be ruled out and the proper direction to train investigation would be to attempt to prepare antibodies from various cell-proteids or nucleic acids prepared as pure as possible. The former point we still have in mind. As Beebe states, from the analysis of his preparations it is evident that the products employed by him are not true nucleoproteids but nucleins, and the molecule of the so-called protein nucleate possesses relatively less of the proteid and more of the nucleic acid radical. He also mentions that the color proteid reactions were given in only a feeble way. Certainly it would seem that the results obtained by the use of such products should be ascribed more probably to the nucleic acid than to the proteid radicals.

The products prepared by us contained less phosphorus than his, pointing to a higher proteid content, and gave characteristic results when tested with Millons, biuret, Adamkiewicz, and Molisch reactions. These reactions could not be ascribable to cell-globulins, which were removed in the process of preparation.

It seems rather dangerous to draw conclusions as to the intravenous clotting obtained when products prepared from organs containing blood are injected in the blood stream. The possibility is always at hand of the presence of fibrin ferment, thrombin, nucleohiston, or whatever may be the agent active in setting about the changes which result in the formation of fibrin in the blood. It is, we think, at present questionable whether it has yet been definitely shown that nucleoproteid, absolutely free from contamination of blood in the general sense, will cause intravenous clotting.

The preparation of the nucleic acids.—The method employed for the preparation of the nucleic acids from the pancreas and the kidney of the dog was essentially that of Levene. The finely comminuted gland was boiled for two hours in a 5 per cent solution of sodium chloride after which sodium acetate in the proportion of 7 per cent of the weight of the fresh glands was added and the mixture allowed to cool. Sodium hydroxide was next added in excess and the material allowed to stand over night. After the reduction of the greater part of the alkalinity with acetic acid the faintly alkaline mixture was precipitated with an excess of picric acid and acetic acid carefully added until a well-formed flocculent precipitate settled out. This filtered quickly. The amount of acetic acid necessary at this point varies according to the kind of nucleic acid. The proteid-free filtrate was next thrown in a large volume of 95 per cent alcohol, and allowed to stand until the nucleic acid settled out. The major part of the alcohol could be removed by siphonage and the precipitate collected on paper. The precipitate was again dissolved in alkali in the presence of sodium acetate and acidulated with acetic acid. A slight precipitate was filtered off. To the filtrate saturated with sodium chloride was added hydrochloric acid until the mixture reacted acid to congo red when an equal volume of alcohol was added. This precipitate showed itself to be biuret free and was dehydrated with alcohol and ether. The products contained from 8 to 9 per cent phosphorus. As was the case with the nucleoproteids, the necessary amount of the substance was dissolved in an appropriate amount of 0.5 per cent sodium carbonate and injected within a few hours after preparation.

DETAIL OF EXPERIMENTS AND RESULTS.

In all instances the dogs received in a small branch of the femoral vein a uniform dose of 2 c.c. of serum per kilo of body weight. The injections were made under light ether anesthesia and this experiment terminated by bleeding from the femoral artery under light chloroform anesthesia. In each group animals were killed at intervals of four, eight, and ten days. Five animals received dog's kidney nucleoproteid serum;* five, dog's pancreas nucleoproteid serum; four, cow's

* In preparing the various sera, the average amount of dog's kidney nucleoproteid to each injection was 0.11 gram; of the dog's pancreas nucleoproteid, 0.13 gram; of the cow's pancreas nucleoproteid, 0.15 gram; of the dog's kidney nucleic acid, 0.25 gram; and of the dog's pancreas nucleic acid, 0.11 gram

pancreas nucleoproteid serum; three, dog's kidney nucleic acid serum; and three, dog's pancreas nucleic acid serum.

Histology.—In no instance did postmortem examination reveal definite macroscopic lesions. The histological study was limited to the kidney, pancreas, and liver. Tissues were fixed in formalin and in Zenker's fluid; the former were examined for fat by the frozen section and Scharlach R method, the latter were embedded in celloidin and stained by hematoxylin and eosin. Fatty changes were not constant, and, when present, of but slight degree. In the kidney nucleoproteid series, fatty changes were found in the kidney series once (10th day) and in no other organ. In the dog's pancreas nucleoproteid series very slight fatty changes were found in three—in one, in liver, kidney, and pancreas; in one, in liver and kidney; in the third, in kidney only. The cow's pancreas series gave negative results by this method. On the other hand, in the nucleic acid series fatty changes in the kidney were constant and well marked, affecting uniformly the loops of Henle. All the other organs were negative except for slight changes in the liver in one animal of the kidney nucleic acid series.

In the study of the Zenker hardened material, in no instance were changes in the pancreas found. The liver, although devoid of well-marked lesions, such as necrosis, almost constantly exhibited a peculiar change affecting all parts of the organ. This condition, which has been noted frequently by one of us as the result of the injection of various cytotoxins, and which unquestionably is that described by other investigators as a granular, vacuolar, or fatty degeneration is characterized by a swollen, pale, "washed-out" appearance of the liver cells with a few large, coarse granules and large irregular pale areas which stain but faintly or not at all. The latter areas are not of the character of fat vacuoles, but more irregular, vary considerably in size, and have no definite relation to the nucleus. The appearance is similar to that of liver cells containing glycogen, and as the same condition in marked degree was found in the adrenal, we are inclined to consider it as an abnormal accumulation of glycogen. Unfortunately this explanation did not occur to us until near the close of our experiments and proper routine tests were not made. Upon the basis of a single attempt to isolate the glycogen

from the last dog of the kidney nucleic acid series, it can be stated, however, that a greatly increased amount of glycogen was present. This was determined by extracting the finely divided gland with hot water and filtering. The filtrate appeared milky white, gave the iodine reaction for the glycogen, which could be precipitated with alcohol at 65 per cent. No particular haste was employed in getting the organ into boiling water and the fact that the glycogen remained so long postmortem points either to a large excess of the compound or perhaps to the absence or great diminution during life of the enzyme in the liver which transforms the glycogen.

The changes in the kidney were purely degenerative in character, of slight degree, and almost constantly present. The cells of the tubules, especially of the convoluted tubules, were swollen and very granular with more or less definite reticulum. As a rule the nucleus could be readily seen, but in the more marked conditions it stained poorly. Rarely the entire lining of the tubule could be seen separated from the basement membranes and lying free in the lumen as a poorly staining granular ring. In addition to these changes the tubules of the cortex, and less frequently, the straight tubules contained the finely granular reticulum so commonly associated with the milder disturbances of the kidney. In the straight tubules this material was often so compressed as to suggest finely granular casts, but definite casts were found but twice and only in the kidney nucleoproteid series. In the collecting tubules and especially in the loops of Henle, in some instances, a more or less marked vacuolization was present corresponding to the fatty change determined by the use of Scharlach R. In no kidney were glomerular changes found.

It is difficult to distinguish sharply between the types of kidney lesions caused by the different sera. In general the changes due to nucleoproteid sera were more severe and widespread than those due to the nucleic acid sera. The latter were characterized by a greater degree of fatty change and by a tendency to involve the loops of Henle rather than the convoluted tubules. If the presence of casts be taken as a criterion the lesions due to the dog's kidney nucleoproteid must be judged the most severe. It is a matter of considerable importance that the cow pancreas serum, in no sense

specific, caused lesions in the kidney not to be distinguished from those due to other sera.

Examination of the urine.—The animals, fed on dog biscuit, were kept in cages suitable for the collection of their urine. This was examined daily for proteids and reducing carbohydrates. The clear filtrated urine was heated to boiling and the merest trace of acetic acid added. This indicated in a roughly quantitative way the amount of coaguable proteids (albumin and globulin) present. It is almost a constant occurrence to find traces of such appearing at variable and irregular times in dog's urine. By traces we mean that upon the addition of acetic acid a faint turbidity appears, which is in no sense a precipitate, but which in the course of 24 hours settles out at the bottom of the tube as a small light fluffy cloud. A second portion was tested with potassium ferrocyanide and acetic acid. The clear urine acidulated with the acid was treated with one drop of the dilute reagent. It was the unusual occurrence to find this test absolutely negative in the dogs under examination in this series of experiments and this result has been confirmed in the examination of many of the urines of other animals at different times. The urines of dogs, concentrated as it is in the passage of an excessively long system of tubules, carries down with it from the collecting tubules not inconsiderable quantities of proteids of the mucin and nucleo-albumin type, which show themselves by the latter test. At times the result of this reaction amounted to a definite flocculent precipitate. With the possible exception of the urines of two of our dogs injected with kidney nucleoproteid anti-serum we feel assured that at no time did our tests give evidence of an excretion of proteid which could in any sense quantitatively or qualitatively be ascribed to changes in the kidney.

In the case of Dog 14 injected on May 20 the urine collected on the 24th showed with heat and with potassium ferrocyanide a decided flocculent precipitate which would be called a positive reaction. The same is true for Dog 15 injected on the same day, the urine reacting positively to both tests on the 25th. Other than these, no results were obtained which would compare in any way to that described by Beebe, where 53 per cent of the urinary nitrogen appeared in the form of albumin. The quantity of proteid in the

cases cited was so small that no attempt was made to determine whether it was an albumin or globulin. At no time were casts obtained in the urine, but upon histological examination of the kidney of Dog 15 as in one other dog (17) of this series, a few hyaline casts were seen in the tubules.

In a paper read this year before the American Medical Association by Robertson and Hacker, and based upon work done in this Laboratory, attention was called to the frequent appearance in human urines tested by Fehling test solutions, of a yellowish-green coloration which varies in intensity from the merest opalescence to a definite, finely divided precipitate which in the course of some hours settles out slowly to the bottom of the tube. It was then stated that this reaction is the result of the addition of too much urine in performing the test and must undoubtedly be ascribed to the presence in the urine at times of some weakly reducing compounds capable in large quantities of partially reducing copper solutions. This pseudo-reaction never occurs when there is employed a small amount of urine, sufficient, however, to show dextrose if present. In testing the urine of dogs these remarks are extremely to the point. When 10 or more drops of the filtered dog's urine were added to the two or three times diluted and boiling Fehling's solution and the mixture boiled a few seconds, it was not at all uncommon to notice in a few moments the appearance of a greenish-yellowish haze which gradually became more opalescent and opaque and which, in extreme cases, might resolve itself into a greenish or yellowish precipitate settling to the bottom of the tube, sometimes immediately, or, more frequently, if left standing, over night. This reduction is not due to dextrose; the urine showed no difference before or after fermentation, polariscopically; no osazone could be obtained, and Nylander's test resulted negatively. If on the other hand, only two or three drops of the urine were added to the diluted Fehling's solution in the ordinary way and not boiled after the addition of the urine the test remained negative. All of the urines tested for dextrose in the latter way, during the course of our experiments, showed absolutely negative results, while the pseudo-reaction was obtained more or less irregularly in 16 of the 20 dogs studied. In our opinion this pseudo-reaction has no direct bearing on the subject under discussion.

In the case of one dog a continued pseudo-reaction was obtained which could be explained by the elimination of lactose following the removal of puppies during lactation. The negative results of Fehling's test with the urine of this animal indicated beautifully the fact contended for in the paper alluded to above, that Fehling's test performed as there advised can be employed without hesitation as a criterion for the presence of dextrose as against other reducing bodies. In none of the urines examined in these experiments was dextrose present in amounts which could be said to exceed the normal, granting that traces of reducing compounds, perhaps dextrose, do appear ordinarily.

While we cannot of course state positively, still it must be granted that it seems extremely likely that the reduction which Beebe describes as a "partial reduction of Fehling's solution" with "the formation of an abundant green precipitate" was nothing more or less than the pseudo-reduction which we have so frequently observed in the urines of normal dogs. As the manner in which his tests were performed or semi-quantitative details are not outlined, such explanations must be classed as only suggestive but not positive. It is our experience that the urines of the dogs injected with dog's pancreas nucleoproteid antiserum show no different character, as regards the content of reducing carbohydrates, than those treated with any of the other sera.

CONCLUSIONS.

In so far as conclusions from so small a number of experiments are justifiable, our results do not support the theory that specific cytotoxic sera may be developed by the injection of nucleoproteids but indicate, rather, that such sera have certain mildly toxic properties acting in a general way and affecting especially the principal excretory organ, the kidney. In this regard we are in accord with Marrassini and in a general way with Bierry and his associates, but in direct conflict with Beebe's very definite statements.

It is clearly evident, however, that Beebe's claims for a specific pancreas serum based on the demonstration of reducing substances in the urine, especially in the absence of histological changes in the pancreas, is untenable in view of our experience with like reduc-

substances more or less constantly present in the urine of normal dogs.

We can make no definite statement concerning nucleoprotein hepatotoxic serum, as we did not prepare such, but it is difficult to explain the focal necrotic lesions of the liver, caused by Beebe's hepatotoxin, as a result of the action of a substance which should, presumably, affect the liver cells more diffusely. It may or may not be a matter of importance that his nucleoproteins were prepared from organs but roughly washed in running water and, therefore, especially as regards the liver, containing large amounts of blood. That a very toxic nucleoprotein may be obtained from red blood cells is well known and it is possible that, as a result, Beebe's serum may have contained hemolysin and agglutinin due to this substance, in addition to the supposed hepatotoxin, in sufficient amount to cause focal liver necroses through agglutinative thrombi, as has previously been described by one of us.⁸

Beebe states that all his sera "are markedly hemagglutinative and hemolytic *in vitro* in dilution up to one to five, but in dilution of one to twenty or above no difference is to be detected between the immune and the normal serum." The hemolytic and hemagglutinative powers of our nucleoprotein sera (the nucleic acid sera were not tested) were much weaker. Thus the dog's pancreas nucleoprotein serum, for example, had very slight power to hemolyze or agglutinate red cells. Two parts of the pure serum to one dog's blood caused neither hemolysis nor agglutination, while 10 parts were required to cause but a faint hemolysis; with the same amount a very definite fine agglutination was evident only after five minutes. It would appear, therefore, by comparison, that the use of blood-free organs in the preparation of nucleoprotein sera lead to the production of a serum less hemotoxic than those developed by the use of unwashed organs. Under such circumstances, therefore, it is very important that future investigators of nucleoprotein sera work with blood-free tissues.

The changes in the kidney resulting from the administration of the kidney nucleoprotein serum, while very definite, cannot be considered as specific or even "special," for similar changes, often of the same grade, characterized the action of all other sera, even that

prepared from cow's pancreas. These changes are, therefore, to be regarded as the effect of the excretion of toxic substances rather than as the manifestation of a selective action of the serum. It must be admitted, however, that it is difficult so to explain the severe albuminuria described by Beebe.

Finally, attention must once more be called to the importance, in all investigations of cytotoxic sera, of the use of blood-free organs, to the occasional occurrence of copper-reducing compounds in the urine of apparently normal dogs, to the not infrequent spontaneous nephritis in these animals, and to the possibility of albuminuria due to a catheter cystitis.

REFERENCES.

1. BEEBE, S. P. Cytotoxic Serum Produced by the Injection of Nucleoproteids, *Jour. Exper. Med.*, 1905, 7, p. 733.
2. PEARCE, R. M. Concerning the Specificity of the Somatogenic Cytotoxins, *Jour. Med. Res.*, 1904, 12, p. 1.
3. ———. An Experimental Study of Nephrotoxins, *Univ. Penn. Med. Bull.*, 1903, 16, p. 217.
4. PORTIS, N. M. Experimental Study of Thyrotoxic Serum, *Jour. Infect. Dis.*, 1904, 1, p. 127.
5. WOLTMANN, H. A Study of the Changes in the Blood and Blood-Forming Organs Produced by Cytotoxic Sera, *Jour. Exper. Med.*, 1905, 7, p. 119.
6. MARRASSINI, A. Recherche sopra l' azione tossica dei nucleoproteidi de fegato, *La clinica moderna*, 1903, No. 13. Reference in *Biochem. Centralbl.*, 1903, 1, p. 805.
7. BIERRY, H. Recherches sur les néphrotoxines, *Compt. rend. de la Soc. de Biol.*, 1903, 55, p. 476.
BIERRY, H., and PETTIT, A. Sur le pouvoir cytotoxique des certains sérums consécutif à l'injection de nucléoprotéides, *ibid.*, 1904, 56, p. 238.
BIERRY, H., and MAYER, A. Sur l'action du sang rendu hépatotoxique par injections intrapéritonéales de nucléoprotéides du foie, *ibid.*, p. 1016.
8. PEARCE, R. M. The Experimental Production of Liver Necroses by the Intravenous Injection of Hemagglutinins, *Jour. Med. Res.*, 1903, 12, p. 329; *ibid.*, 1906, 14, p. 541; Experimental Cirrhosis of the Liver, *Jour. Exp. Med.*, 1906, 8, p. 64.

THE STREPTOCOCCI FROM SCARLATINAL AND NORMAL THROATS AND FROM OTHER SOURCES.*

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INTRODUCTION.

It is a well-established fact that streptococci are quite constantly found in great abundance on the tonsils of scarlet-fever patients. This is shown very clearly by the investigations of Weaver, Baginsky and Sommerfeld, Charlton, Charbade, Booker, Tangl, Klein, Kurth, and others. It is also well known that these organisms are frequently found on the tonsils of healthy persons, although not in such great abundance as on the tonsils of scarlet-fever patients. Hilbert, Tunnicliff, Dungen, Schweighofer and Widal, and Bezancon state that they found them in all normal throats which they examined. Others, however, did not find them so constantly. Black found them on only 30 per cent of the normal tonsils which he examined. Netter found them in 5.5 per cent; Kurth in 8 per cent; Podbielsky in 2 per cent; and Dornberger in 45 per cent of the normal throats examined by them. In most of these investigations no detailed study was made of the streptococci that were found. In some investigations glucose-broth tubes were inoculated with material from the tonsils, and if cocci were found in chains after incubation of the tubes, it was concluded that streptococci were present. Apparently no further study was made of the cultures. In consideration of this fact it was thought worth while to take up this question again and make a more detailed study of the different strains of streptococci and diplococci which can be isolated from scarlatinal and normal throats.

METHOD OF STUDY.

Through the researches of Schottmüller¹ and of Rosenow² we know that pneumococci produce green colonies in blood-agar plates, while *Streptococcus pyogenes* produces small grayish colonies which

* Received for publication August 2, 1906.

¹ *Munch. med. Wchnsch.*, 1903, 50, p. 909.

² *Jour. Infect. Dis.*, 1904, 1, p. 308.

are surrounded by a perfectly clear area of hemolysis. Schottmüller also pointed out the fact that there are streptococci which produce green colonies in blood-agar plates, very similar to the pneumococcus colonies. In this study I have made use of these facts in isolating *Strept. pyogenes* from material obtained from the tonsils. A sterile cotton swab was rubbed against the tonsils in such a manner as to avoid the tongue so much as possible. The swab was then rinsed in 1-1.5 c.c. of sterile broth, and the latter was used immediately to inoculate three or four blood-agar tubes and the same number of litmus inulin-agar tubes which were poured into plates and incubated for 24 to 48 hours at 36° C. Blood-agar was prepared by melting plain agar tubes, cooling to 45° C., and adding 0.5 c.c. of sterile defibrinated rabbit or human blood to each tube. Litmus inulin-agar was prepared as previously described.¹

After 24 hours' incubation it is possible to pick out the streptococcus colonies in the blood-agar plates as they are small gray colonies surrounded by a perfectly clear area of hemolysis. It must be remembered, however, that diphtheria bacilli and pseudodiphtheria bacilli may produce hemolyzing colonies which closely resemble the streptococcus colonies. Furthermore, I have occasionally found pneumococcus colonies which produce considerable hemolysis and may therefore be mistaken for *Strept. pyogenes* colonies. The pneumococcus colonies are not easily recognized in these plates as there are always found in the throat many chain-forming cocci which form green colonies like the pneumococcus colonies. In all instances subcultures were made from four to eight hemolyzing colonies and the same number of green colonies. These cultures were studied morphologically and culturally and their fermentative power was tested in Hiss's² inulin serum-water medium, or in a slightly modified medium which was prepared as follows:

Dissolve 5 gr. of NaCl, 20 gr. of peptone (Witte) and 20 gr. of pure inulin in 1,000 c.c. of distilled water. Add 20 c.c. of a 5 per cent solution of pure litmus and tube, putting 2 c.c. of the mixture into each tube, and sterilize in the autoclav. After sterilization, add (with a sterile pipette) 2 c.c. of sterile heated ascites fluid,* or preferably heated beef serum, to each tube and incubate for 24 hours before using.

¹ *Jour. Infect. Dis.*, 1906, 3, p. 183.

² *Jour. Exper. Med.*, 1905, 6, p. 317.

* Great care must be observed not to use ascites fluid which contains fermentable carbohydrates. Each lot must be tested with organisms that are known to have great fermentative powers and if acid is produced it must not be used.

The beef serum was collected without taking any special precautions but was diluted with an equal volume of water and passed through a large Berkefeld filter, drawn off into 50 to 100 c.c. tubes and heated to 65° C. for one half-hour on two successive days.

This medium was preferred to Hiss's serum-water because it was found that some pneumococci and some streptococci which have recently been obtained from blood cultures do not grow well in the latter.

In the early part of this work no litmus inulin-agar plates were used, but pneumococci were looked for among the green colonies in blood-agar plates. In those cases subcultures were made and studied from 10 to 12 green colonies. Organisms were classed as typical pneumococci only: (1) When they were found to be Gram-positive cocci which grew chiefly in pairs on blood-agar slants; (2) when they fermented inulin; and (3) produced green or slightly hemolyzing colonies in blood-agar plates.

In the litmus inulin-agar plates most pneumococci produce red colonies which are easily recognized on the blue background, and as practically no other mouth bacteria produce red colonies in this medium¹ it is easy to isolate pneumococci from the mouth and throat with the aid of these plates. Subcultures were, however, always made from two or three red colonies, and the organisms positively identified. In no instance have I isolated an organism by this method which could not be classed as a pneumococcus, although some of these organisms form chains of considerable length in some liquid media, and they do not seem to have a well-formed capsule.

RESULTS.

A total of 154 throat cultures were made and examined as follows: Normal 51; scarlatinal 75; measles 14; tonsilitis 5; pneumonia 5; and laryngitis 4. The findings differ very widely and must be considered under separate heads.

Scarlatina.—All the blood-agar plates inoculated with material from scarlatinal throats contained many *Strept. pyogenes* colonies and varying numbers of green colonies. The cases may be roughly divided into 3 great groups: (1) Those from which the plates contained mostly (60 to 95 per cent) *Strept. pyogenes* colonies —31 cases. Two

¹ *Jour. Infect. Dis.* 1906, 3, p. 183.

sets of plates contained only *Strept. pyogenes* colonies. (2) Those from which the plates contained about as many *Strept. pyogenes* colonies as green colonies—20 cases. (3) Those from which the plates contained fewer *Strept. pyogenes* colonies (15 to 40 per cent) than green colonies—21 cases. As a rule the streptococcus colonies greatly predominate over the other colonies when the inflammation of the throat is pronounced, and they rapidly decrease in number with the subsidence of the throat symptoms. When the throat symptoms are mild the proportion of *Strept. pyogenes* colonies to the other

colonies is often quite small. I have met with one exception to this statement in the case of a small girl who had a very bad throat, but the plates inoculated with material from it contained far more green colonies than *Strept. pyogenes* colonies. A large proportion of the green colonies in this set of plates were typical pneumococci which proved to be highly virulent for rabbits.

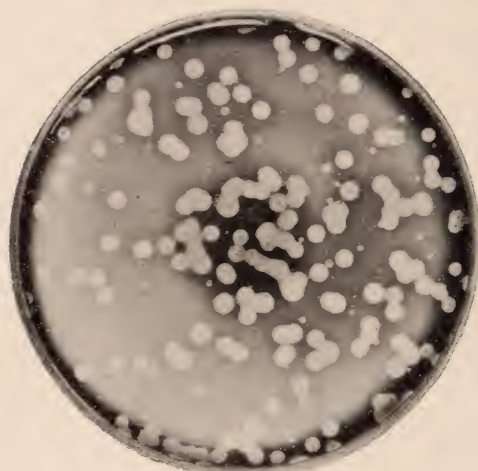


FIG. 1. — Photograph of a blood-agar plate inoculated with material from a scarlet-fever throat. Many *Strept. pyogenes* colonies and few small green colonies.

Pneumococci were not looked for in four cases but were found in 64 of the 71 throats that were examined for them—91.4 per cent. Diphtheria bacilli were found four times and *B. mucosus* and *B. influenzae* once. *Staph. aureus* and *citreus* and *M. tetragenus* were found occasionally, but these were probably contaminations from the tongue. Gram-negative diplococci which form small brownish colonies were found quite frequently. These were not always studied in detail, but some were identified as *M. catarrhalis*.

Normal throats.—All blood-agar plates inoculated with material from normal throats contained many green and slightly hemolyzing greenish colonies. *Strept. pyogenes* colonies were found in 30 of 51

throats which were studied, but were never present in large numbers (see Fig. 2) and were entirely absent in 21 cases (41.2 per cent). In only five sets of plates did I find more than 10 per cent of *Strept. pyogenes* colonies, and in 15 sets there were only 1 per cent or less of these colonies. Not infrequently one finds hemolyzing colonies in blood-agar plates which, upon closer study, are found to be organisms that produce greenish colonies in subsequent plates. Great care must therefore be exercised in deciding on the presence or absence of *Strept. pyogenes* colonies. The slightly hemolyzing, often greenish colonies also are very puzzling and will be described more fully on a later page. Pneumococci were found 42 times (82 per cent), and diphtheria bacilli three times. Gram negative diplococci and staphylococci were found quite frequently and *B. mucosus* once.

Measles throats.—The findings in the throats of measles patients corresponded closely to those of normal throats.

Strept. pyogenes was found in 9 of 14 throats which were examined (65 per cent) and pneumococci were found in 12 (85.7 per cent).

Tonsillitis.—*Strept. pyogenes* colonies always predominated in these plates, but green colonies also were present. Pneumococci were found in four of the five cases examined, but were not specially looked for in the other case. In one case influenza bacilli were found in great abundance.

Pharyngitis.—Four cases of so-called pharyngitis were examined, but none of the blood-agar plates inoculated with material from these throats contained *Strept. pyogenes* colonies. All plates contained many green colonies and some contained small brownish colonies.

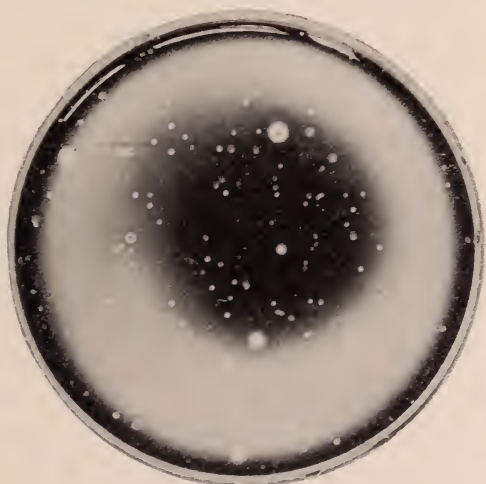


FIG. 2.—Photograph of a blood-agar plate inoculated with material from a normal throat. Many small greenish colonies and four *Strept. pyogenes* colonies.

The latter were chiefly Gram negative cocci, but in one case they were influenza bacilli. Pneumococci were isolated from all of these cases. The finding of pneumococci in such a large per cent of throats of persons not afflicted with pneumonia is in agreement with the work of the Commission for the Investigation of Acute Respiratory Diseases of the Health Department of the City of New York.¹

Pneumonia.—Three of the five sets of plates inoculated with material from the tonsils of pneumonia patients contained very few *Strept. pyogenes* colonies, and the other two sets did not contain any. All blood-agar plates contained many green colonies and the litmus inulin-agar plates contained many red colonies. Typical pneumococci were easily isolated from all plates.

It will be noticed that green colonies were found in all blood-agar plates except two sets which were inoculated with material from scarlatinal throats. Only a small proportion of these colonies are pneumococci and the remainder form a large group of organisms between the typical pneumococci and streptococci. Some of these organisms seem to correspond to Schottmüller's *Strept. viridans*, but others are very closely related to the pneumococcus. In addition to the green colonies there are often found slightly hemolyzing colonies which may sometimes have a green tint. These colonies, which are more abundant in plates inoculated with material from normal throats and from throats of measles patients than in plates inoculated with material from scarlatinal or tonsilitis throats, must not be confounded with the *Strept. pyogenes* colonies. Rarely one finds chain cocci in throats which form hemolyzing colonies in plates of plain agar and blood, and green colonies of the pneumococcus type in glucose blood-agar plates. These organisms I have found in the throats of two measles patients and once in the throat of a healthy man. All these organisms, including the *Strept. viridans*, are closely related to one another and form a great group which can be sharply differentiated from the *Strept. pyogenes* by the following characteristics:

1. The cocci found in smears made from milk cultures stain rather poorly and unevenly with Loeffler's methylene blue, are usually somewhat elongated or lance-shaped and often are found in pairs

¹ *Jour. Exper. Med.*, 1905, 7, p. 401.

in the chains. On the other hand, *Strept. pyogenes*, when grown in litmus milk, takes the methylene blue stain very readily, the

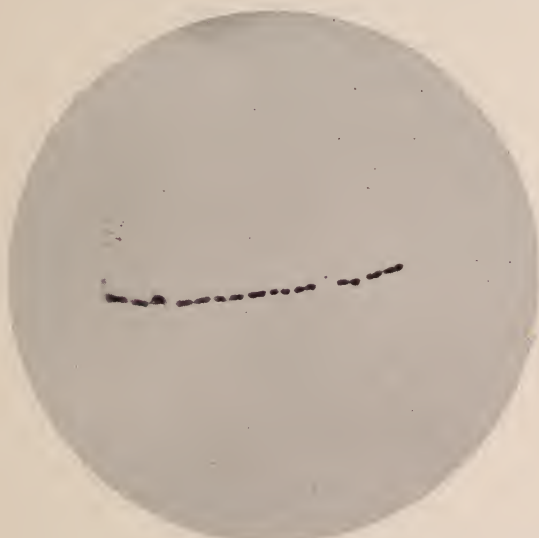


FIG. 3.—*Strept. viridans*. Cocci are elongated and arranged in pairs in the chain. Microphotograph $\times 1,500$.

and the chains have the appearance of having been stretched. This grouping in pairs in the chains is shown in Fig. 3.

2. On ascites-agar slants a large proportion of these atypical organisms form lance-shaped or oval cocci which are grouped chiefly in pairs, but also in short chains. A grouping in pairs within the chains is usually observed. Some of the cocci have well-formed

cocci are never distinctly grouped in pairs in the chains, and are not elongated, but frequently appear to be disc-shaped. On account of this peculiarity the cocci in the chains of *Strept. pyogenes* appear closely packed together, while those of *Strept. viridans* and allied organisms are relatively far apart



FIG. 4.—*Strept. pyogenes*. Cocci are not elongated and are not arranged in pairs in the chains. Microphotograph $\times 1,500$.

capsules and might be classed as pneumococci, although they do not ferment inulin and usually form chains in liquid media.

3. When pure cultures of these organisms are grown on serum-glucose-agar slants (three parts of glucose agar and one part of ascites fluid or serum) no change is produced in the medium, while *Strept. pyogenes* turns it white and opaque in 36 to 48 hours. This interesting reaction was first observed by Libman¹ who used it to differentiate the pneumococcus from *Strept. pyogenes*. Libman's observation is correct as far as it goes, but it does not go far enough, because there is a large group of cocci which cannot be classed as pneumococci although their behavior on the serum-glucose-agar is like that of typical pneumococci. It must be stated, however, that most of these organisms are more closely related to the pneumococcus than to *Strept. pyogenes*.

Something over 1,500 milk cultures of different strains of chain-forming cocci from throats and other sources were examined microscopically and classed either as *Strept. pyogenes* or as atypical forms. Seventy-five strains which had thus been identified as typical *Strept. pyogenes*, 77 strains which had been classed as atypical forms, and 16 strains of pneumococcus were planted on serum-glucose-agar slants and incubated for three days. The results are shown in Table 1. Seventy-four of the 75 strains of *Strept. pyogenes* turned the medium white and opaque in 48 hours, while only four of the atypical forms and one pneumococcus produced a similar change in three days. Three of the four atypical forms which changed the culture medium were isolated from the same throat (normal). These produce slight hemolysis in blood-agar plates and readily ferment inulin, which indicates that they are not typical *Strept. pyogenes*. The fourth strain forms green colonies in blood-agar plates, the cocci are round, often arranged in pairs, in chains of 8 to 16 cocci, and have a well-formed capsule.

The fact that some of the atypical forms produce green colonies while others form hemolyzing colonies in blood-agar plates may suggest to some that they are very different organisms. This does not necessarily follow, because I have shown that the green coloration is dependent on the production of acid and the action of this

¹ *Jour. Med. Res.*, 1901, 1, p. 84.

TABLE I.

THE EFFECT OF *Streptococcus pyogenes*, PNEUMOCOCCI, AND CULTURES OF INTERMEDIATE COCCI ON SERUM-GLUCOSE-AGAR.

Streptococcus pyogenes.

Source	No. of Races Tested	Character of Colonies in Blood-Agar Plates	Effect on Serum-Glucose-Agar
Scarlet-fever throats.....	29	Hemolyzing	White opacity
Measles throats.....	4	"	"
".....	1	"	No change
Normal throats.....	18	"	White opacity
Tonsilitis.....	2	"	"
Otitis media.....	4	"	" "
Erysipelas.....	4	"	" "
Hand infection.....	4	"	" "
Puerperal sepsis.....	4	"	" "
Meningitis.....	2	"	" "
Conjunctivitis.....	1	"	" "
Phlegmon of the leg.....	1	"	" "
Suppurative lymphadenitis.....	1	"	" "
Frontal sinus infection.....	2	"	" "
Pericardial fluid (body dead of scarlatina).....	1	Hemolyzing	White opacity

ATYPICAL FORMS.

Scarlet-fever throats.....	8	Green	No change
" " " ".....	2	Hemolyzing; greenish	" "
" " " ".....	1	Green	White opacity
Measles throats.....	1		No change
" " " ".....	1	Green in glucose-blood-agar; hemolyzing in plain-blood-agar plates	" "
Tonsillitis.....	3	Green	No change
Normal throats.....	18	" "	" "
" " " ".....	30	Slightly hemolyzing; not green	" "
Normal throats.....	7	Hemolyzing; greenish	No change
" " " ".....	1	Green in glucose-blood-agar; hemolyzing in plain-blood-agar plates	" "
Normal throats.....	3	Slightly hemolyzing	White opacity
Otitis media (mixed infection).....	2	Green	No change

PNEUMOCOCCI.

Normal throats.....	4	Hemolyzing greenish	No change
" " " " " " " " " "	4	Green	" "
Scarlet-fever throats.....	1	" "	White opacity
Conjunctivitis.....	1	" "	No change
Blood cultures from pneumonia patients	4	" "	" "

acid on the red corpuscles.¹ When the culture secretes hemolysin rapidly the red corpuscles about the colony are destroyed early and no green color is seen even if a considerable amount of acid is produced. On the other hand, some cultures of *Strept. pyogenes* which produce hemolyzing colonies in plain blood-agar plates form green colonies in plates of glucose-agar and blood. Furthermore, the secretion of hemolysin by bacteria is a variable character. It is very pronounced in some strains of streptococcus, rather feeble in others,

¹ *Jour. Infect. Dis.*, 1906, 3, p. 663.

and may be absent entirely in others. I have two strains of streptococcus which have lost this property entirely by being grown in glucose broth for about two years. They now produce brownish colonies in plain blood-agar plates and greenish colonies in plates of glucose-agar and blood.

THE FERMENTATIVE POWER OF STREPTOCOCCUS PYOGENES

The fermentative power of 124 races of *Strept. pyogenes* from various sources was tested on the following carbohydrates: dextrin, inulin, lactose, maltose, raffinose, rhamnose, saccharose, and salicin; and on the polyatomic alcohols mannit and dulcit. The culture medium used was that described on p. 756 except that the carbohydrate and peptone solutions were sterilized by the fractional method* before the addition of the serum. Sterile serum which had been heated to 65°–70° C. was added to each tube after sterilization. In this medium all strains fermented dextrin, lactose, maltose, saccharose, and salicin, but none fermented inulin, raffinose, rhamnose, and dulcit. Occasionally a strain would not readily ferment dextrin or saccharose, but on repeated trials it would ferment them. These substances, therefore, did not reveal any differences between the various races of streptococcus used.

The hexatomic alcohol, mannit, however, was found to be fermented quite readily by some races and not at all by others. This substance was used then to divide the various races of *Strept. pyogenes* into two classes, (1) those which ferment mannit and (2) those which do not ferment it. The chief points of interest are shown in Table 2. Nearly all these cultures were obtained from a single colony in a blood-agar plate which had been inoculated with material from an infected region. In many instances subcultures from two or three colonies in the same plate were tested, and it was found that these results always agreed. In no instance did I get two strains from the same infection which did not have the same effect on mannit. Five strains were tested 3 times, at intervals of 2 to 3 months, and the results were always the same. The table therefore represents less than one-half of the tests that have actually been made.

* It is sometimes almost impossible to sterilize some of these carbohydrates by the fractional method. After a great many failures I hit upon the scheme of sterilizing twice on the third day. This makes four sterilizations with only a short space of time between the last two. By this method I hardly ever failed to get every tube sterile.

TABLE 2.

CLASSIFICATION OF STREPTOCOCCI ACCORDING TO THEIR EFFECT ON MANNIT.

Source of Streptococci	No. of Races Tested	Mannit Fermented	Mannit not Fermented
Scarlatinal throats.....	60	37	23
Normal throats.....	19	6	13
Measles throats.....	6	1	5
Tonsilitis throats.....	6	3	3
Phlegmon of the leg.....	3	1	2
Erysipelas.....	7	4	3
Otitis media (post-scarlatinal).....	6	4	2
Otitis media (measles).....	1	..	1
Hand infection.....	4	..	4
Pericardial fld. (body dead of scarlatina).....	2	2	..
Suppurative lymph-adenitis (complicating scarlatina).....	1	..	1
Suppurative lymph-adenitis (complicating erysipelas).....	2	1	1
Mastoid abscess.....	1	..	1
Frontal sinus infection.....	1	1	..
Meningitis.....	1	..	1
Conjunctivitis (scarlatinal).....	1	1	..
Cervix uteri (puerperal sepsis).....	1	1	..
Blood culture (puerperal sepsis).....	1	1	..
Arm infection.....	1	..	1
Totals.....	124	63	61

To obtain uniform results it is necessary that the culture medium should always be prepared in the same manner. If the nutritive qualities of the culture medium are allowed to vary one can hardly expect to get uniform results. A number of preliminary tests with Hiss's serum-water medium which had been sterilized at 100° C. showed a considerable lack of uniformity. This was thought to be due to the fact that some of the cultures grew rather poorly in this medium.

Although these tests show a difference in the fermentative power of different races of *Strept. pyogenes*, it will be noticed that there is no agreement between the source of the culture and its effect on mannit. Further differences between the organisms of these two classes were now looked for by means of agglutination tests with immune serum.

AGGLUTINATION.

Three sheep were immunized with different strains of streptococci as follows: Sheep "A" with a mannit-fermenting and Sheep "B" with a non-fermenting *Strept. pyogenes*; Sheep "C" with a *Strept. viridans*. The sheep were injected every 10 days, for a period of four months, first with heated cultures and later with living cultures, until 30 c.c. of a 48 hour calcium broth culture was borne without bad effects. Agglutination tests with the sera of these

sheep showed that not all mannit-fermenting streptococci are agglutinated by the serum of the sheep which had been immunized with a mannit-fermenting organism, while some of the non-fermenters are agglutinated by that serum. Similarly, not all non-fermenters are agglutinated by the serum of the sheep which was immunized with the non-fermenting organism. None of the strains of the *Strept. pyogenes* are agglutinated by the serum of the sheep immunized with the *Strept. viridans*.

All tests were macroscopic, using 72 hour calcium broth cultures according to the method described by Hiss.¹ The tubes were incubated for three hours and then set into the ice chest for 12 hours before recording the results. The details are shown in Table 3.

The tests show, moreover, that many strains of *Strept. pyogenes* coming from scarlet fever are agglutinated by the serum of that sheep (Sheep "A") which had been immunized with a streptococcus isolated from a scarlet-fever throat. These organisms are not agglutinated by the serum of Sheep "B" which was immunized with a streptococcus isolated from a phlegmon of the leg. Of the 11 strains which were obtained from scarlet-fever patients, three (381, Laura, and Morris) were not agglutinated by the serum of Sheep "A" in higher dilutions than by the serum of Sheep "B."

TABLE 3.
AGGLUTINATION OF STREPTOCOCCI BY
1. SERUM OF SHEEP "A."

STREPTOCOCCI	DILUTIONS				
	1:50	1:100	1:200	1:300	1:500
Mannit Fermented—					
Frances.....	+++	+++	+++	+++	+++
Anna.....	+++	+++	+++	++	++
Duffy.....	+++	+++	+++	+++	++
381.....	+	o	o		
Smyth.....	o	o	o		
Goldspohn.....	+++	++	+		
Stuart.....	+++	++	+		
Mannit not Fermented—					
Coburn.....	+	o	o		
Morris.....	o	o	o		
Geragass.....	++		o		
Joe R.....	+++	+++	++	++	+
Henry R.....	+++	++	+	o	
Laura.....	o	o	o		
Harris.....	++	+	+	o	
Mintz.....	+	o			
C. T. N.....	++	++	o		
G 2.....	o	o	o		

¹ Jour. Exp. Med., 1905, 7, p. 560.

2. SERUM OF SHEEP "B."

Mannit Fermented—					
Frances.....	o				
Anna.....	o				
Duffy.....	o				
381.....	+				
Smyth.....	o				
Goldspohn.....					
Stuart.....	++	++	+	o	
Mannit not Fermented—					
Coburn.....	+++	+++	+++	+++	+++
Morris.....	o				
Geragass.....	+	o			
Joe R.....	o				
Henry R.....	o				
Laura.....	o				
Harris.....	o				
Minte.....	o				
C. T. N.....	o				
G 2.....	o				

3. SERUM OF SHEEP "C."

Mannit Fermented—					
Frances.....	o				
Anna.....	o				
Duffy.....	o				
381.....	+				
Smyth.....	o				
Goldspohn.....	o				
Stuart.....	++	+			
Mannit not Fermented—					
Coburn.....	o				
Morris.....	o				
Geragass.....	o				
Joe R.....	o				
Henry R.....	o				
Laura.....	o				
Harris.....	o				
Minte.....	o				
C. T. N.....	o				
G 2.....	+++	++	+		

Sheep "A" immunized with *Streptococcus* Frances.

Sheep "B" immunized with *Streptococcus* Coburn.

Sheep "C" immunized with *Strept. viridans* (G 2).

Source Streptococci "Frances," "Joe R.," "Henry R.," "Harris," from scarlet-fever throats; "Duffy" and "381" from pericardial fluid of scarlet-fever bodies; "Anna," "Goldspohn," and "Laura" from scarlatinal otitis media; "Smyth" from uterus in puerperal sepsis; "Stuart" from erysipelas; "Coburn" from phlegmon of the leg; "Morris" from suppurating lymph gland in scarlatina; "Geragass" from abscess in erysipelas; "C. T. N." from tonsil in tonsilitis; "G 2" is a *Strept. viridans* from a scarlet-fever throat.

Organisms "381" and "Laura" had each passed through more than 15 rabbits. Organism "Morris" was obtained from suppurating cervical glands of a very mild case of scarlatina. The cervical adenitis did not appear until the end of the fourth week, after the patient had been allowed to be out of bed for more than two weeks.

The serum of Sheep "B" agglutinated only the homologous organism.

It is not necessary here to go into the literature on agglutination of streptococci, but mention should be made of the work of Ross-wall and Schick¹ who found that Moser's antistreptococcus serum agglutinated many strains of streptococcus coming from scarlet-

¹ *Wiener klin. Wchnschr.*, 1905.

fever patients. They call special attention to an organism obtained from a case of surgical scarlatina which was agglutinated by Moser's serum in high dilutions. From the throats of some scarlatinal patients they isolated streptococci which were agglutinated by that serum and some that were not agglutinated by it. It must be recalled that Moser's serum is prepared by immunizing horses with streptococci that are isolated from scarlet-fever patients, without passing the organisms through animals.

VIRULENCE OF STREPTOCOCCI AND PNEUMOCOCCI ISOLATED FROM THROATS.

Most cultures of *Strept. pyogenes* and some pneumococci isolated from scarlatinal and normal throats have some virulence for rabbits, but *Strept. viridans* has practically no virulence for these animals. Thirty-one rabbits weighing from 900 to 1,200 G. were injected intraperitoneally with *Strept. pyogenes* cultures as follows: Four rabbits with a suspension of two 48 hour blood-agar slant cultures of organisms isolated from normal throats. Three of these animals died and one remained well. Twelve rabbits, with the same kind of a suspension of organisms isolated from scarlatinal throats. Eight of these animals died and four remained well. Eight rabbits, with a suspension of one blood-agar slant culture of organisms isolated from normal throats. Five of these animals died and three remained well. Seven rabbits, with a suspension of one blood-agar slant culture of organisms isolated from scarlatinal throats. One of these animals died and six remained well.

These results indicate that streptococci isolated from normal throats are slightly more virulent for animals than those isolated from scarlatinal throats. This is in perfect agreement with the results obtained by Hilbert¹ and Tunnickliff.²

Strept. viridans has a lower virulence for rabbits than *Strept. pyogenes*. Nine rabbits weighing from 900 to 1,200 G. were injected into the peritoneum with two blood-agar slant cultures of different strains of these organisms, but only one of these animals died.

Ten rabbits were injected with two blood-agar slant cultures of different strains of pneumococci isolated from normal and scarlatinal throats. Four of these animals died and the other six remained

¹ Zeitschr. J. Hyg., 1899, 31 p. 381.

² Trans. Chicago Path. Soc., 1904, 6, p. 175.

well. Fourteen rabbits were then injected with larger doses, but only five of these died. There are no indications that pneumococci in scarlatinal throats are more virulent than those in normal throats, with the one exception referred to above.

CONCLUSIONS.

Strept. pyogenes is constantly found in great abundance on the tonsils of patients suffering from tonsilitis and scarlet fever, before the inflammation of the throat has subsided. These organisms rapidly decrease in numbers with the subsidence of the throat symptoms.

Strept. pyogenes cannot be considered a normal inhabitant of all healthy throats, although it was found in small numbers in 59 per cent of the normal throats in this series.

Pneumococci of low virulence were found in 119 of this series of 154 throats.

A large group of organisms which lies between the typical *Strept. pyogenes* and the pneumococcus was found in all normal throats and in nearly all diseased throats. These organisms have very little virulence for rabbits, and, as they are found in great abundance in practically all throats, they appear to be normal inhabitants of the throat. These organisms can be sharply differentiated from *Strept. pyogenes* both morphologically and by their effect on serum-glucose-agar.

Cultures of *Strept. pyogenes* can be divided into two classes according to their fermentative power: (1) those which ferment mannit, and (2) those which do not ferment it. Not all inulin-fermenters are agglutinated by the serum of a sheep immunized with an inulin-fermenting streptococcus, while some non-fermenters are agglutinated by it. Most streptococci coming from scarlet-fever patients are agglutinated in fairly high dilutions by the serum of a sheep immunized with an organism of that source.

Strept. pyogenes from normal throats appears to have a slightly greater virulence for rabbits than when obtained from scarlatinal throats.

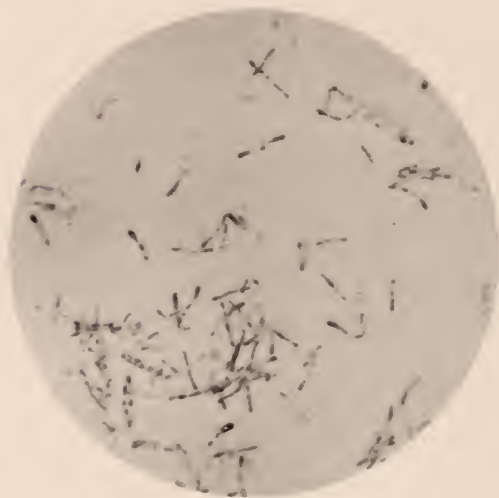
I am indebted to Professor Hektoen for many suggestions and to Dr. W. L. Baum for the privilege of getting throat cultures from patients under his care at the Cook County Hospital.

A NEW SIMPLE METHOD FOR STAINING THE POLAR BODIES OF DIPHTHERIA BACILLI.*

ALBERT A. EPSTEIN.

(From the Pathological Laboratory of Mt. Sinai Hospital, New York.)

BLUMENTHAL and Lipskerow, in their study of the comparative values of all the different methods devised for staining the polar bodies of diphtheria bacilli, came to the conclusion that the Ljublinski-



pyoktanin method gives the best results.¹

But they found that even with this method difficulty is at times experienced in that it fails to give distinct pictures of the bacillus proper, although the polar bodies may be stained very well. This deficiency in the staining of the entire organism constitutes an objection to the use of this method be-

cause it renders the presence of tingible cocci a possible source of error. My own experiences with the various polar-body stains compel me to concur with the view of these authors.

Often a well-ripened solution of Loeffler's alkaline methylene blue will define fairly well the morphological characteristics of the diphtheria bacillus. This definition may be greatly enhanced by the subsequent use of Gram's iodine solution as a differentiating agent. Better results are to be obtained, however, by the use of a solution of pyronin, followed by the iodine solution.

* Received for publication June 30, 1906.

¹ *Centralbl. f. Bakt.*, 1905, Orig., 38, p. 359.

The solutions necessary for the purpose are:

1. Loeffler's alkaline methylene blue or a 1 per cent aqueous solution of pyronin (preferably the latter).
2. Gram's iodine solution.

The steps in the method are as follows:

- I. Pyronin solution for 20 seconds, or Loeffler's solution for $\frac{1}{2}$ minute.
 - II. Rinse in tap water.
 - III. Gram's iodine solution for 10 seconds.
 - IV. Rinse in tap water, dry, and examine.
- Counterstains are not required.

With either of these methods the polar bodies and the body of the bacillus appear very sharply defined and their relation to each other is very definitely shown. With the pyronin solution the polar bodies appear as large, dark, brick-red, round, or oval bodies, whereas the bacillus is usually slender and of a light red color. In some instances the polar bodies contain an unstained refractile central spot.

With the Loeffler solution the polar bodies appear greenish-black and the bacillus of a greenish color. In both instances the entire organism is very distinct and the contrast between the polar bodies and the bacillus proper is striking; but the beauty of the pictures which result with the use of pyronin should make it the method of choice.

The accompanying photomicrograph (1,000 diameter magnification) shows quite well the appearance of diphtheria bacilla stained by the pyronin method. The original slides show the polar bodies even more distinctly.

A SIMPLE METHOD OF STERILIZING BLOOD FOR CULTURAL PURPOSES.*

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(From the Pathological Laboratory Mt. Sinai Hospital, New York.)

THOUGH great attention has been given of late to blood media, the necessity for a method of preparation which would make such media more widely applicable has been felt. Much of the difficulty hitherto experienced has been that of obtaining sterile blood readily and in quantities sufficient for universal use. This difficulty compelled various investigators to select widely different animals for their source of supply. Some even resorted to the use of commercial hemoglobin. The work, therefore, was varied and no standard blood media could be established.

After a series of experiments we have been able to devise a method by which the difficulties previously mentioned may be overcome. Numerous attempts have been made in the past to sterilize the blood to be used for cultural purposes, but of the methods so far introduced there is none which permits of sterilization without at the same time altering the character of the blood.

For the purpose of obtaining unchanged blood upon which the (so-called) hemolytic and other effects of bacterial growth could be determined, we resorted to the use of formalin as a sterilizing agent. We found that formalin could be used for this purpose without later exerting any inhibitory influence on the growth of bacteria. In order to prevent clotting of the blood ammonium oxalate was employed. The ease with which beef blood can be obtained in large quantities in any abattoir led us to the selection of this source of supply.

As a result of our experiments we are prepared to present the following method as being the most satisfactory:

Four hundred c.c. of beef blood are drawn directly into a sterile Erlenmeyer flask of 500 c.c. capacity containing 30 c.c. of a 1 per cent solution of ammonium oxalate (in distilled water) and $\frac{1}{2}$ c.c. of for-

* Received for Publication June 30, 1906.

malin of 40 volume strength. The flask is then shaken for one or two minutes. Thirty minutes is the time required for the sterilization of the blood which is then transferred in small quantities into sterile Erlenmeyer flasks and diluted with twice its volume of sterile (0.9 per cent) saline solution. This dilution reduces the actual formalin content to one part in 2,400 of blood.

According to our experiences it is best to allow this diluted blood to stand for 24 to 48 hours at room temperature before employing it for cultural purposes so as to allow for better diffusion of the formalin. The blood at this stage must receive the same care as any other sterile nutrient medium. The flasks are then sealed and stored on ice until needed.

In the preparation of our media we used the diluted blood in the proportion of 1 part to 15 of nutrient material (agar or broth). This quantity of blood we have found to be the optimum for showing character of growth, color changes, and hemolysis. The final product has an absolute formalin content of 1 part in 36,000. The ordinary laboratory organisms are found to grow very well on these media and luxuriant growths of the pneumococcus, meningococcus, gonococcus, and influenza bacillus are readily obtained. Full details of various observations will appear in a later publication.

Throughout the entire procedure of sterilizing the blood there are no evidences of any gross or microscopic changes. It remains of the same scarlet color as when first drawn and the blood corpuscles are unaltered. This permits of the separation of the blood corpuscles from the serum which, if so desired, may be accomplished by centrifugalization or by allowing the blood to stand and washing the sedimented corpuscles in several changes of sterile salt solution. No doubt media prepared with washed corpuscles and those prepared with the entire blood will be found to give interesting cultural differences. In the use of the former any possible bactericidal effect of the serum would be eliminated.

It is necessary to state in this connection that an occasional beef will yield a blood which is of a deep crimson color. Such blood should not be selected for the work as laking has occurred which naturally interferes with the study of hemolysis.

MOUTH DISINFECTION IN THE PROPHYLAXIS AND TREATMENT OF PNEUMONIA.*†

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INTRODUCTION.

MOUTH cleansing has long been practiced, but the full significance of it has been recognized only in recent years since it has been known that the secretions of the mouth contain large numbers of bacteria; some pathogenic and such as give rise to serious infectious processes, others non-pathogenic but associated with deleterious changes affecting the teeth and other tissues. Further study has now shown that virulent bacterial species are present in the secretions of healthy as well as diseased individuals, and not occasionally, but frequently. Thus as a means of controlling the spread of this infectious material, mouth cleansing has come to be more and more seriously considered, not only by physicians, but by the educated public. Recently, in the hope of securing disinfection, simple cleansing has to a large extent been replaced by a variety of antiseptic procedures which have been adopted without much definite knowledge of their efficiency. The general problem has been studied, but these researches, for the most part conducted with faulty technic or based solely on clinical observation, have not, except in diphtheria, been applied specifically to the study of different diseases. Accordingly, to determine the practical value of mouth disinfection in the prophylaxis and treatment of pneumococcus infection, these studies were undertaken in the hope of securing accurate data of specific import, and thus of being able to discriminate definitely between the more efficient and the inefficient, even harmful, procedures of which many have been widely recommended and are extensively used.

* Received for publication August 6, 1906.

† This study was carried on under the auspices of the Medical Commission for the Investigation of Acute Respiratory Diseases of the Department of Health of the City of New York.

THE PRACTICAL PROBLEM OF MOUTH DISINFECTION.

The bacteria of the mouth are continually changing;* large numbers are swallowed; others are thrown off in expectoration, and still others degenerate in the secretions or are taken up by the phagocytes and destroyed. On the other hand, the supply is being constantly replenished from the inspired air and ingested food, by the drainage of the nares and larynx through the mouth and pharynx, and also by the growth of species which develop in this environment.

The mucous membrane and anatomical arrangement of the mouth and pharynx are well adapted for the elimination of many of the bacterial species which gain access to the secretions, but which are unable to develop in this unfavorable environment. They are also equally well adapted for the protection and harboring of certain other species which grow and become established in the secretions as harmless parasites.† It is these bacteria, usually pathogenic although of varying grades of virulence, which are the most serious menace to health and the most troublesome in the attempts at mouth disinfection.

In the nares conditions are similar to those of the mouth, but the nasal secretion is chiefly mucus, and is rarely diluted with food or drink or by other secretions. Apparently harmless parasitism occurs less frequently in these secretions.‡ In the trachea, the secretions are practically pure mucus; and bacteria disappear with astonishing rapidity. It would thus appear that aside from many significant, but for present purposes indeterminate, conditions affecting the elimination, development, or protection of bacteria in this as in other parts of the body, mucus is peculiarly important.

* Rose's³² observations show the fluctuations under normal conditions during the 24 hours; the largest number of bacteria were found before breakfast, after the night's quiet; the smallest number after each meal; following this a gradual increase up to the next meal.

† Netter³⁴ demonstrated the presence of pneumococci in the mouths of pneumonia patients 10 or more years after recovery, and found these organisms much more frequently in cases which gave a history of pneumonia.

‡ Witness, for example, the researches of Thomson and Hewlett,³⁶ who determined experimentally the rapid disappearance of the bacteria deposited on the nasal mucous membrane. Neumann²⁵ was able to find pneumococci in the nasal secretion of only 4 per cent of the normal cases examined, whereas in the mouth, observers have rarely failed to obtain these organisms in at least 20 per cent of the normal cases. von Besser's³ comparative results also confirm these findings.

Fresh mucus* has uniformly proved in the experience of several observers, distinctly bactericidal, and it is significant that the mucous membranes† which have a pure mucous secretion are usually free from microorganisms although they are more or less exposed to contamination. Furthermore, mucus diffuses slowly with foreign material and as a natural bland protection to the mucous membrane there is no adequate substitute. In the nares, pharynx, trachea, and mouth, irritation of the mucous membrane is immediately followed by an outpouring of fresh mucus; a flushing from within outward by a natural secretion which apparently meets all the requirements of an ideal wash.

Thus it is that the natural resources of the tissues under normal conditions are extremely well adapted for the protection of the individual carrying virulent pathogenic bacteria in the mouth as harmless parasites. One may well hesitate before disturbing this nice and well-perfected arrangement of nature, but conditions arise in disease when it is evident that these natural resources may be greatly aided in re-establishing the normal equilibrium between the bacteria on the one hand and the susceptibility of the tissues on the other. And then, although for the time being themselves adequately protected, healthy individuals if they carry about in their secretions dangerous bacteria, may become a menace to their fellows.

In the attempt to aid the natural resources of the tissues in the elimination of infectious material by mouth cleansing or disinfection, there are several factors which render the problem both serious and difficult. The mucus which protects the mucous membrane from the bacteria also protects the bacteria from the action of foreign substances. The removal of mucus exposes the mucous membrane to more or less irritation which may be accompanied by exudation, development of the bacteria remaining in the secre-

* In the studies of Wurtz and Lemoyez³¹ with sputum, of Walther³² and of Stroganoff³⁴ with cervical secretion, and of Arloing¹ with the secretion of mussels, the bactericidal qualities of mucus have been determined *in vitro*. Grawitz,³³ Steffen,³⁵ and also Hopkins,¹⁵ failed to detect this action, but in their studies the material was sterilized by heat. Recently, Wood¹⁰ found that pneumococci were much more quickly destroyed in mucus than in sputum.

† The uterine and the greater portion of the urethral and bronchial mucous membranes on careful examination have usually under normal conditions proved sterile. The introduction of bacteria in the animal experimentation of Morisani²³ on the uterus, of Beco² and others on the respiratory tract, was followed under normal conditions by a rapid disappearance of these organisms. My own experiments with the pneumococcus in the respiratory tract indicate that these organisms under normal conditions disappear from the tracheal and bronchial mucous membranes of rabbits with astonishing rapidity.

tions with increase of virulence, and possibly infection. Many antiseptics are irritants and in the mouth of doubtful disinfecting value. Solutions to be used in the mouth and pharynx should therefore be selected with care and should possess certain essential qualities. In general, they should be bland to the mucous membranes, diffuse quickly with the secretions, and, if antiseptic, should be of known practical value.

Most observers in recent years advise approximately neutral reactions for mouth washes. Acids have been used. Bacteria in the fermentation of sugars form acid rapidly and when certain percentages are reached growth stops and some species, especially the pneumococci, die rapidly. On the other hand, caries of the teeth Miller²⁰ has shown to be largely due to the acid fermentation of sugars in the mouth by bacteria,* and it is now deemed better to neutralize whatever acid may be present rather than to introduce any more. Alkalis have also been recommended, chiefly on account of the more rapid diffusion with the secretions, but they have little or no action on the bacteria in strengths which can be used with comfort in the mouth. On the gums and teeth they are generally considered injurious. The reaction of solutions to be used in the mouth should therefore be neutral or preferably slightly alkaline to neutralize whatever acid may be present.

Since many of the complicated solutions fail to do more than remove the contaminated secretion, the use of plain water has been recommended. The body fluids and particularly those containing mucus, do not, however, diffuse well with water and the tissue cells undergo varying degrees of plasmolysis. Although these are not serious objections to the use of water in the mouth, better results it is well known may be secured if the water be made isotonic by the addition of salts.

Antiseptics obviously should be not only efficient in destroying the bacteria, but bland to the mucous membrane, and if by chance swallowed or absorbed, harmless to the individual. The choice is thus limited. Nevertheless a great variety of antiseptics have been recommended by different observers. When such diversity

* The recent investigations of Monier²² and of Rodella³⁰ suggest that anaerobic bacteria are largely responsible for this sugar fermentation.

of opinion exists and when similar results are secured by different procedures, the efficiency of the antiseptics may be seriously questioned. In fact, the researches conducted in the New York Health Department under Dr. Park showed that in a long series of diphtheria cases the best results were secured by cleansing the mouth with simple normal salt solution.

In the early experiments of Miller,²¹ and in the recent studies of Rose,³¹ portions of the mucous membrane were cleansed and remained sterile for a few minutes; but it was impossible even to approximate complete disinfection of the mouth with any of the long list of antiseptics tested. Miller²¹ recommended bichloride solutions. Rose³¹ condemned them and recommended in turn 60 per cent alcohol used with a brush as the most efficient antiseptic in the mouth. Other observers apparently have obtained excellent results with alcohol and advise its use in the mouth, but not stronger than 30 per cent, because in higher percentages its continued use desiccates and hardens the tissues. Although the antiseptic value of alcohol has long been recognized,* it may be of interest to note in this connection that students of hand disinfection† have recently come to look upon it as one of the most useful and efficient disinfectants employed in solving the intricate and difficult problems encountered in this field of investigation. The unsubstantiated claims and recommendations of other observers‡ concerning other antiseptics, many of which have already proved in Miller's or Rose's experiments inefficient, might be cited, but to no purpose.

It thus appears that the results of former studies agree in but one particular, that complete disinfection of the mouth is impossible. With the methods employed no discrimination can be made between the part played by normal physiological processes aided by simple cleansing and the part played by antiseptics in the elimination of bacteria. Obviously, the antiseptics could not be expected to accomplish the impossible, but apparently no adequate effort was made to determine their action under favorable conditions, and in view of the conflicting results, the varied recommendations, and the

* For recent studies see the work of Weigl¹⁸ and Wirgin.³⁰

† Witness, for example, the researches of Engels,⁸ Flecksedor,¹⁰ Shaffer,³² Sturtz,³⁵ Bonhoff,⁵ Danielshon and Hess.⁷

‡ *Vide*, Beck in Kolle and Wassermann,¹⁶ also Ritter,²⁰ Miller,²¹ Rose,³¹ and Grewe.⁴³

substantial support given simple measures, it might well be assumed that many of the experiments were conducted with solutions antiseptic in name only or in lower dilutions.

THE PRACTICAL PROBLEM OF MOUTH DISINFECTION IN PNEUMONIA.

The problem of getting rid of the pneumococci in the secretions of the mouth is beset with even greater difficulties. With the possible exception of the streptococci, the pneumococci of all the pathogenic bacteria are most apt to become established as harmless parasites. Extremely susceptible to environmental change, and readily adapting itself to grow in serous exudates, the pneumococcus often rapidly acquires an exalted virulence. In the mouth the secretions are often diluted and contain nutrient material; and the pneumococci develop and persist under these conditions to such an extent that they are to be found in some individuals constantly, in others frequently, and in crowded communities at one time or another in nearly everyone.* In the purely mucous secretions of the healthy intact mucous membranes the pneumococci, like other species of bacteria, do not long survive. It is thus probable that comparatively few of the pneumococci develop in the glandular openings where the fresh mucous secretions are continually being poured out. The folds of mucous membrane about the teeth and tonsils, and in other inaccessible places, on the other hand, doubtless harbor considerable numbers of these organisms.†

Under these conditions and in the light of the experience of previous observers, it might reasonably be inferred that the problem of securing the elimination of the pneumococcus is quite as impossible as that of complete disinfection. Nevertheless, by securing a mouth-wash of definite antiseptic value, and then by continually removing and destroying the pneumococci in the more accessible parts of the mouth, it was hoped and thought possible that in healthy individuals at least the outpouring of fresh secretion might gradually replace the contaminated secretion until finally few, if any, pneumo-

* The results of Park and Williams,²⁷ Longcope and Fox,¹⁸ Buerger,⁹ and Hiss²⁴ are of particular significance as they suggest the conditions prevailing in our large cities during the winter months.

† Bezançon and Grignon² on scraping the mucous membranes of the tonsil found pneumococci in 100 per cent of the cases examined, exceptional results as compared with those obtained in examinations of other observers.

cocci remained. But the first step was to determine accurately and from a practical view-point the most efficient solution to be used in the mouth. This, it may be recalled, should react neutral or slightly alkaline, diffuse rapidly with the secretions, and be isotonic and bland to the mucous membranes, and not poisonous. Finally, if antiseptic, it should also and above all be efficient.

Antiseptics vary in their mode of action: some combine chemically with the bacterial cell or coagulate the proteids; others give rise to plasmolytic changes. The pneumococcus, it was thought, owing to its albuminous capsule, and its great susceptibility to change of environment as compared with other species of bacteria, might differ in its susceptibility to these different antiseptics. It was accordingly deemed wise to determine first the comparative susceptibility of different pathogenic bacteria to plasmolysis, and then secure accurate data regarding the action of various antiseptic solutions on the pneumococcus under different conditions. Finally, a study of the practical value of the most efficient of the antiseptic solutions as compared with that of simple isotonic salt solution, was conducted on healthy individuals whose secretions contained virulent pneumococci, and on pneumonia patients.

PLASMOLYSIS OF PATHOGENIC BACTERIA IN SODIUM CHLORIDE SOLUTION.

For comparison with the pneumococcus, the bacilli of diphtheria, anthrax, pneumonia (Friedländer), and typhoid, the vibrio of cholera, and *Staphylococcus pyogenes aureus*, were selected. From 24 hour agar cultures of these organisms, broth emulsions were made and $\frac{1}{2}$ c.c. transferred to test-tubes containing 2 c.c. of the various salt solutions.* The appearance of the tubes was noted at stated intervals. At the same time definite quantities were plated in agar and counted after 24 or 48 hours' incubation. The results of this experiment appear in the following table:

* The solutions were made as follows:			
25 per cent NaCl in H ₂ O		Glucose 1 per cent, Peptone 1 per cent in H ₂ O	
5 c.c.		5 c.c.	15 c.c.
10		5	10
20		5	0
0		5	20
			= 5%
			= 10
			= 20
			= Control

PLASMOLYSIS OF THE BACILLI OF DIPHTHERIA, ANTHRAX, PNEUMONIA, AND TYPHOID, THE *VIBRIO* OF CHOLERA, AND THE *STAPHYLOCOCCUS PYOGENES AUREUS*, IN SODIUM CHLORIDE SOLUTIONS.

	CONTROL		5 PER CENT NaCl			10 PER CENT NaCl			20 PER CENT NaCl			
	2 Hrs.	9 Hrs.	2 Hrs.	9 Hrs.	30 Hrs.	2 Hrs.	9 Hrs.	30 Hrs.	2 Hrs.	9 Hrs.	20 Hrs.	30 Hrs.
<i>B. diph.</i>	0 4.982 0	0 227,868 floc. growth ∞	0 76,320 0	0 72,404 0	0 12,077 growth	0 63,600 0	0 14,046 0	0 600 0	0 2,650 0	0 001 0	0 0 0	0 0 0
<i>B. anth.</i>	0 11,448 0	0 growth ∞	0 45,792 0	0 113,335 0	0 growth ∞	0 63,600 0	0 52,788 0	0 0	0 1,400 0	0 1,310 0	0 0 0	0 0 0
<i>B. Fried.</i>	0 0	0 growth marked ∞	0 0	0 growth marked ∞	0 0	0 286,200 0	0 77,502 ?	0 50,880	0 70,074 0	0 13,091 0	0 0 0	0 0 0
<i>B. typh.</i>	0 0	0 growth marked ∞	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 174,000 0	0 0 0	0 0 0
<i>V. chol.</i>	180,035 0	0 growth marked ∞	83,520 0	124,024 growth marked	?	0 0	0 growth marked	0 0	0 0	0 growth	0 0 0	0 0 0
<i>Staph. p. a.</i>	∞	∞	∞	∞	∞ +	∞ + +	∞ +	∞ + +	∞ + +

EXPLANATORY NOTE.—A = appearance of the tube; O, indicating no change. C = actual count estimated per c.c.; ∞, indicating that the colonies were too numerous to count; +, representing degree of positive findings; — no observation; and 0, a sterile plate.

In the development of the colonies there was often a marked inhibition which could not be conveniently shown in the table. The transfers from the stronger solutions and the transfers after longer exposures required longer incubation for the development of the colonies. This varied directly with the exposure and the strength of the solutions. Furthermore, the size of the colonies was similarly influenced. These phenomena were not observed in the plates of the staphylococcus; they were most marked in the plates of the anthrax, cholera, and Friedländer organisms.

These tests suffice to indicate the wide differences in the susceptibility to the plasmolysis of sodium chloride solutions that may exist among different species of pathogenic bacteria.* The insusceptibility of anthrax spores is shown by the constant figures obtained in the counts from the strong 20 per cent solutions. On the other hand, anthrax vegetative cells, quickly destroyed in the 20 per cent solutions, showed slight development in the 10 per cent solutions, and were thus slightly more susceptible than the diphtheria bacilli, and considerably more so than the Friedländer and typhoid organisms. The staphylococcus, which was little affected by the 20 per cent solutions, proved the most resistant. The cholera vibrio, quickly destroyed in the 10 per cent solutions, was the most susceptible. Attention may also be directed to the fact that although the Friedländer organism is an encapsulated cell, similar to the pneumococcus, it was one of the least susceptible to plasmolysis.

In the experiments with the pneumococcus, definite quantities from the tests were not plated in agar and counted as in the experiments with the other species of bacteria. The method is particularly unfavorable for the growth of the pneumococcus and many cells fail to develop under these conditions. Streaking the surface of serum agar offers the most favorable conditions for the growth of the pneumococcus. As this method was considered a more reliable and significant test of complete disinfection, it was adopted in all the following experiments. In the simple glucose peptone-water media used in making the solutions of the previous experiments, the pneumococcus would not grow. A more favorable meat infusion broth was therefore substituted.

PLASMOLYSIS OF THE PNEUMOCOCCUS IN SODIUM CHLORIDE SOLUTIONS.

24 Hr. Broth Cul. Pneumo.	25 % NaCl, H ₂ O	0.85 % NaCl	Media	Approx. % NaCl 37°	½ Hr.	3 Hrs.	10 Hrs.
5 c.c.	1 c.c.	9 c.c.	0 c.c.	2.5%	+	+	+
5	3	0	7	5.7	+	dim.	0*
5	5	0	5	8.9	+	"	0*
5	10	0	0	17.0	+		0*

* Smears on fresh media failed to show growth; the streaks were, therefore, sterile.

Similar results were obtained in other experiments with the pneumococcus. Cultures concentrated by centrifugalization were sub-

* Of late, plasmolysis of bacterial cells in weak solutions of salts and alkalis have been extensively studied, especially in its relation to the lysis observed in immune sera; *vide* Fischer.⁹ The action of strong salt solutions on some of the saprophytic bacteria has recently been studied by Lewandowsky.¹⁷

jected to strong salt solutions; exposure to 8 or 10 per cent in 12 hours nearly always killed the organisms; even lower percentages often sufficed, but occasionally a few living cells were found which were doubtless in the clumps or masses of cells and precipitates formed in broth cultures and were thus protected from the action of the salt solution. In some instances these living cells were virulent, as shown by the development of infection in animals inoculated with these cultures.*

It is thus evident from these studies on the plasmolysis of sodium chloride solutions that as compared with other species of pathogenic bacteria, the pneumococcus cell, aside from its capsule, is extremely susceptible, being destroyed on exposure to comparatively weak (6 per cent) solutions.

PLASMOLYSIS OF THE PNEUMOCOCCUS IN ALKALINE SOLUTIONS.

The marked susceptibility of the pneumococcus to the osmotic changes of sodium chloride solutions suggested tests of the action of alkalis, particularly as these have been used in mouth-washes.

PLASMOLYSIS OF THE PNEUMOCOCCUS IN ALKALINE SOLUTIONS.

		85% NaCl	Pnc. Cult.	Alkali %	37° 10 Min.	1½ Hrs.
NaHCO ₃ 5 % in { H ₂ O	5 c.c.	0 c.c.	5 c.c.	2.5%	+++	+ very few colonies
	3	2	5	1.5	+++	++
	1	4	5	0.5	+++	+++

		85% NaCl	Pnc. Cult.	Alkali %	37° 15 Min.	30 Min.	17 Hrs.
LiCO ₃ 1% in { H ₂ O	5	0 c.c.	5 c.c.	0.5%	+++	+++	0
	3	2	5	0.3	+++	+++	0
	1	4	5	0.1	+++	+++	0
	0.5	4.5	5	0.05	+++	+++	+
LiCO ₃ 1% in { H ₂ O					37° 20 Min.	4 Hrs.	
	5	0 c.c.	5 c.c.	0.5	+++	0	
	3	2	5	0.3	+++	0	

NOTE.—Growth on the plates is indicated by the plus (+) sign; sterile plates. 0.

* Gilbert and Carnot¹² found the virulence of pneumococci growing in culture deteriorated rapidly when much smaller percentages were added to the media. My results thus seemingly fail to corroborate their findings. It may be that this is due to the different methods of experimentation, or Gilbert and Carnot's results may have been due to other conditions, particularly as the changes in virulence of pneumococci growing in artificial media are marked and variable.

The marked action of the lithium salt led to tests of the neutral chloride of this alkali in the hope that it might prove more active than sodium chloride. Lithium chloride, however, failed to give better results than the sodium salt.

The results of these tests show that the pneumococcus is also extremely susceptible to the osmosis of alkaline solutions; and that the alkalis, owing doubtless to their different atomic composition, vary greatly in their action; but that this susceptibility to osmosis is not such as to be of practical value in destroying bacteria in the secretions of the mouth, however useful may be the addition of salts and alkalis in rendering other antiseptic solutions less injurious to the individual or more efficient in their action on bacteria.

THE ACTION OF ANTISEPTICS ON THE PNEUMOCOCCUS.

Bichloride of mercury and carbolic acid combine chemically with albuminous substances, precipitating them from solution; in fact, it is to this property that their antiseptic action is largely attributed. By the chemical union the antiseptic is so weakened, and by the precipitating action suspended bacteria caught in the coagula are protected to such an extent that the efficiency of these antiseptics in albuminous solutions is impaired. As disinfectants of sputum, carbolic acid and bichloride of mercury have proved unreliable and are now rarely used. Aside from this they are both poisons when taken internally. For these reasons neither bichloride of mercury nor carbolic acid were considered of sufficient practical value in mouth disinfection to warrant a study of their action on the pneumococcus.

Various astringent antiseptics at one time much used in the mouth have been discarded and are now considered more harmful than efficacious. The studies were thus limited to what were considered, from a practical view-point, the more promising antiseptics. Potassium chlorate, lysol, formaldehyde or formalin, the peroxide of hydrogen, and alcohol, were selected for the experiments. In order to simplify the work and at the same time secure data for subsequent comparison the tests were made on broth cultures of the pneumococcus.

THE ACTION OF POTASSIUM CHLORATE, LYSOL, FORMALIN, HYDROGEN PEROXIDE AND ALCOHOL SOLUTIONS ON PNEUMOCOCCUS CULTURES

	Salt Sol. 0.85%	Pneu. Cul.	Dil.	5 Min.	25 Min	4 Hrs.
KClO ₃ Sat. H ₂ O..	5 c.c. 0 3 2 c.c. 1 4 5 0	5 c.c. 5 5 5 5 5	½ sat. 1 ½ 1-200 1-1,000	0 0 +	++++ ++++ ++++	++++ ++++ ++++
Lysol 1-100.....	1 qs. ad. 5 0.5 " 5 0.25 " 5 0.1 " 5	5 5 5 5 5 5 5 5	1-2,000 1-4,000 1-10,000	++ +++ +++	0 0 +	
				10 min.	1½ hr.	
Formalin 1-100...	5 c.c. qs. ad. 5 3 " 5 1 " 5 5 " 5	5 c.c. 5 5 5 5 5 5 5	1-200 1-333 1-1,000 1-2,000	+++ +++ +++ ++	0 0 0 +	
1-1,000.....	3 " 5 1 " 5	5 5 5 5	1-3,333 1-10,000	++++ ++++	++ +++	
*Creosote (Beech-wood)	0.02 0 0.01 0	5 5 5 5	1-250 1-500	0 ++		
				5 min.	40 min.	
H ₂ O ₂ (Merck) diluted to 3%....	5 c.c. 0 3 qs. ad. 5 1 " 5 0.5 " 5	5 c.c. 5 5 5 5 5 5 5	1.5 % 1.0 0.3 0.15	0 0 ++ +++	0 0 0 0	
H ₂ O ₂ (Oakland) 3%	5 0 3 qs. ad. 5 1 " 5 0.5 " 5	5 5 5 5 5 5 5 5	1.5 1.0 0.3 0.15	0 0 ++ +++	0 0 0 +	
					1½ hr.	
Alcohol.....	5 c.c. 0 3 2 1 4	5 c.c. 5 5 5 5 5	50 % 30 10	0 0 ++++	0 0 +	
Control.....	5 5	5 5	1-1	++++	++++	

NOTE.—Creosote has been so strongly advocated in the treatment of many infections of the respiratory tract by many observers (*vide* Robinson; *Med. Rec.*, 1906, 69, p. 529) that these tests were made of its action on the pneumococcus. From the results it is evident that Creosote in non-irritating dilutions is not sufficiently antiseptic to warrant its use in mouth washes.

In these experiments on pneumococci in broth culture, potassium chlorate proved inactive, and formalin but slowly active. On this account they were considered of little or no practical value in mouth disinfection. Lysol, hydrogen peroxide, and alcohol, however, were quick disinfectants, but their action in broth could not be accepted as any indication of their action in the mouth where the pneumococci are suspended in serous and mucous secretions. Accordingly, lysol, peroxide of hydrogen, and alcohol were tested as to their action on pneumococci in serous exudates, such as are readily obtained from the pleuritic processes incited by the intrathoracic inoculation of virulent pneumococci in rabbits. Rabbits were inoculated with the exudate containing the antiseptics to determine any loss of virulence in the pneumococci.

ACTION OF LYSOL, HYDROGEN PEROXIDE, AND ALCOHOL ON PNEUMOCOCCUS EXUDATES.

	Salt Solution		Pneu. Exudate	Dilution	15 Min.	1 Hr.	3 Hrs.	4 Hrs.
Lysol 1-1,000.....	2.5 c.c.	qs. ad.	2.5 c.c.	1-2,000	+		+	
	1.25	"	2.5	1-4,000	+		+	
	0.5	"	2.5	1-10,000	+		+	1 c.c. killed rabbit in 48 hrs.
1-100	1.0	qs. ad.	5.0	1-1,000	+	+	+	
	0.5	"	5.0	1-2,000	+	+	+	1 c.c. killed rabbit in 72 hrs.
	0.25	"	5.0	1-4,000	+	+	+	1 " " " 72 "
Control	0	qs. ad.	5.0	0	+	+		1 c.c. killed rabbit in 48 hrs.
	2.5	qs. ad.	2.5	1.5%	+		+	
	1.25	"	2.5	0.75	+		+	
H ₂ O ₂ 3%	0.5	"	2.5	0.30	+		+	
	2.0	qs. ad.	2.5	40	0		0	
	1.5	"	2.5	33½	0		0	
Alcohol	1.25	"	2.5	25	0		0	
	1.0	"	2.5	20	0		0	
	0.75	"	2.5	15	+	+	0	1 c.c. failed to infect rabbit
Control					+		+	1 " killed rabbit in 36 hrs.
			2.5	0	+	+	+	

In these experiments lysol and hydrogen peroxide proved inefficient disinfectants, whereas the action of alcohol was little affected by the albuminous material of the exudate in which the pneumococci were suspended. No loss of virulence was detected in the living pneumococci after four hours' exposure.

The failure of so many of these antiseptics to act on the pneumococcus under the conditions of these experiments suggested that it might be well to study some of the much advertised commercial solutions which contain a variety of antiseptics and are extensively used as mouth-washes. Accordingly, samples of Borine, Borolyptol, Glycothymoline, Listerine, Odol, and tablets made up according to the formula of Seiler, were purchased, and tests similar to those tabulated above were made.

ACTION OF BORINE, BOROLYPTOL, GLYCOTHYMO LINE, LISTERINE, ODOL, AND SEILER'S SOLUTION ON THE PNEUMOCOCCUS.

	Salt Sol.	Pneu. Cul.	Dil.	5 Min.	25 Min.
Borine	5 c.c. 0 1 qs. ad. 4 c.c.	5 c.c. 5	1-1 1-10	0 ++++ 10 min.	0 ++++
Borolyptol	5 1 qs. ad. 5	5 5 5	1-1 1-5 1-10	++ +++ ++++ 5 min.	+ ++ +++
Glycothymolin	5 0 1 qs. ad. 4	5 5	1-1 1-10	++++ ++++	++++ ++++
Listerine	5 0 1 qs. ad. 4	5 5	1-1 1-10	0 ++++	0 ++++
Odol	1 5 0.1 " 5	5 5	1-10 1-100	0 ++++	0 ++++
Seiler's Formula.....	5 0 1 qs. ad. 4	5 5	1-1 1-10	++++ ++++	++++ ++++
Control.....	0 " 5	5	1-1	++++	++++

	Salt. Sol.	Pneumo. Exudate	Dil.	10 Min.	60 Min.
Borine	2.5 c.c. 0 1.5 qs. ad. 1.5 c.c.	2.5 c.c. 2.5	1-1 1-4	++++ ++++	0 ++++
Listerine	2.5 0 1.5 qs. ad. 1.5	2.5 2.5	1-1 1-4	0 ++++	0 0

As shown by the results of these simple experiments, the failure of all these commercial solutions, is so complete as to render unnecessary further study of their inefficiency.*

By conducting the experimental tests of the action of antiseptics on the pneumococcus along these lines—first on broth cultures and

* Some of these and other solutions prepared by commercial houses abroad have also proved inefficient when tested as mouth washes in the experiments of foreign observers; *vide* Pelnav,¹⁸ Miller,²¹ Rose,³¹ and Greve.¹³

then on exudate obtained from infected rabbits—it was possible to simplify the study and exclude from prolonged research a long list of antiseptics. In fact, alcohol alone of all the antiseptics studied gave definite results. But lysol was not studied (in the tests with exudate) in the strongest solutions which the mouth will tolerate, so that the researches were continued with stronger solutions of this substance and alcohol. Alcohol and lysol were combined in the hope that the efficiency of one might be increased by the other. It was now possible to conduct these tests on pneumococci embedded in sputum. The pleural exudate from infected rabbits containing numerous virulent pneumococci was shaken with double the quantity of sputum. In this way an excess of the pneumococci was insured without interfering seriously with the physical or biological properties of the sputum. Sputum, as is well known, is changed physically and biologically by heat and it is impossible to filter out the contaminating bacteria, so the above method was adopted and gave excellent results. Rabbits were inoculated in these experiments to determine any loss of virulence in the pneumococci and also to control the observations made from the streak plates. The results of these tests are tabulated for convenience according to the plan used in the previous experiments.

ACTION OF ALCOHOL AND LYSOL ON PNEUMOCOCCI IN SPUTUM.

Lysol 1-100	Alcohol	Salt Sol.	Pneu. Ex. + Sputum	Dil.	20 Min.*	2½ Hrs.
2 c.c.	3 c.c.	5 c.c.	1-500	++++	++++
1	4	5	1-1,000	++++	++++
2	1 c.c.	2	5	Lysol, 1-500	++++	++
				Alc., 10%		
1	1	3	5	Lysol, 1-1,000	++++	++++
				Alc., 10%		
	3	2	5	Alc., 33½%	o	o
	2	3	5	" 20	+++	++
	1	4	5	" 10	++++	++++
Control	o	5	5	o	++++	++++

Alcohol 25 per cent	H ₂ O	Pneu. Ex. + Sputum	Dil.	15 Min.*	1 Hr.	1½ hours 3 c.c. of Sputum. Which Settled to Bottom of Tube Inoculate Rabbit.
20 c.c.	o c.c.	5 c.c.	20%	++++	++	D. 72 hrs.
15	5	5	15	++++	++++	D. 36 "
10	10	5	10	++++	++++	D. 40 "
Lysol 1-100—						
2.5 c.c.	qs. ad. 20 c.c.	5	1-1,000	++++	++++	D. 36 "
1.25	" 20	5	1-2,000	++++	++++	D. 48 "
Control	" 20	5	o	++++	++++	D. 36 "

* Cultures made from shreds of sputum floating on surface.

In these experiments lysol proved inefficient in dilution of 1-500 and there was no advantage in combining it with alcohol. For complete disinfection of pneumococcus sputum, slightly higher percentages of alcohol were required than for pneumococcus exudate. The virulence of pneumococci in sputum was not affected by either lysol or alcohol.

SUMMARY OF THE EXPERIMENTAL TESTS OF ANTISEPTICS.

From these experimental tests of the action of different antiseptics on pneumococci in broth cultures, in exudates, and in sputum, it is evident that the pneumococcus is much more susceptible to disinfection in broth than in exudates, and that in sputum it is exceptionally well protected, so much so in fact that few substances in bland dilutions have any appreciable action on either the viability or the virulence of these organisms. Hydrogen peroxide,* which quickly destroyed the organisms in broth cultures, proved worthless in the presence of the exudates, and lysol was little better. Of all the solutions studied, alcohol† was the least influenced by the presence of the albuminous material and detritus in the exudates or the mucus in the sputum. In fact little variation in the susceptibility of the pneumococcus could be observed in any of these alcohol tests. The antiseptic action of alcohol, therefore, was attributed not to the coagulation of cell proteids, but to osmotic changes in the pneumococcus cell, which, it may be recalled, proved exceptionally susceptible to the plasmolysis of sodium chloride solutions. Thus it is that alcohol alone withstood the test of these experiments *in vitro*, and alone offered any promise of solving the problem of ridding the mouth of pneumococci by means of antiseptics.

Although the results of these researches *in vitro* were definite, the problem in the mouth is so different and there are so many uncertain factors, that before attempting the final tests on pneumonia patients, studies of the diffusion of alcoholic and other solutions

* Hydrogen peroxide has also been tested in the general problem of mouth disinfection by Rose³¹ who found 5 per cent of no practical value and 10 per cent only in a degree efficient. Rodella³² recommends hydrogen peroxide as the best wash in caries of teeth, but does not give the results of his studies with it in detail.

† The experiments also show that the addition of lysol had only slight effect on the activity of the 10 per cent solution.

with sputum were made in the hope that the practical efficiency of alcoholic solutions might be increased.

STUDIES ON THE DIFFUSION OF ANTISEPTIC AND BLAND
SOLUTIONS WITH SPUTUM.

The various antiseptic, aqueous, alcoholic, salt, and alkaline solutions used in the previous experiments were colored by an anilin dye, carefully pipetted with equal quantities of fresh saliva in test-tubes, and the line of demarkation between the two fluids noted from time to time. Owing to the clumps or shreds of mucus accurate observations could not be made and only the more obvious differences were noted. Of the aqueous solutions the diffusion of the sodium bicarbonate was most marked, but the difference was not great. Similar observations were noted for the alcoholic solutions which were slightly more pronounced than the bicarbonate.

Glycerine it was thought might render solutions more bland to the mucous membranes. Accordingly studies were made of its influence on the diffusion of solutions with saliva or sputum. A mixture of glycerine, alcohol, and water gave the best and quickest results, which were much more marked than with any of the solutions alone.

Diffusion of these solutions at different temperatures was also made and it was found that heat accelerated the diffusion and that hot solutions when used in the mouth seem to leave the mucous membranes cleaner and fresher than cold solutions. Forty-five degrees Centigrade is the highest recorded temperature which the body has attained and at the same time recovered from.* Fluids at this temperature were tried in the mouth and it was found that this was about the maximum which the mucous membrane would bear with comfort.

This maximum temperature (45° C.) at which mouth washes can be used is so much higher than that usually attained by the body tissues and also so much higher than that at which attenuation in cultures of virulent pneumococci has been reported (Fraenkel¹¹) and only 7° below the thermal death-point of the organism (52° C.), that a study of the effect of short exposure to this temperature on viability and virulence was made.

* EVE, *Jour. Phys.*, 26, 1900, p. 119.

EFFECT OF HEAT (45°C.) ON THE PNEUMOCOCCUS.

The blood of a rabbit dying from pneumococcus infection was mixed with broth, and $\frac{1}{4}$ c.c. of this dilution added to tubes containing 1 c.c. of 2 per cent peptone. After an hour's incubation they were exposed in bath to 45° C. Cultures and inoculations into mice were made at stated intervals.

45°	Plate 24 Hrs. Growth	Mouse Inoculation	Result
5 Min.....	++++	Dead in 60 hours	Pneumo. infect.
10 ".....	++++	" " 84 " approx.	" "
20 ".....	++++	" " 84 " "	" "

However valuable for physical or physiological reasons the use of hot mouth washes may be, the temperatures at which such solutions can be used have practically no effect on either the virulence or the viability of the pneumococcus.

SUMMARY OF THE TESTS OF DIFFUSION AND THE VALUE OF HEAT.

The results of these observations on the diffusion of different solutions with saliva or sputum suggest that the addition of sodium chloride and sodium bicarbonate in bland quantities may facilitate the diffusion of these solutions with the secretions, but that the best results are secured when these solutions are used hot. By the addition of glycerin the diffusion of such solutions with the secretions of the mouth is greatly accelerated.

For the practical tests, therefore, the bland simple solutions containing NaCl 0.5 per cent, sodium bicarbonate 0.25 per cent, were used hot. The alcoholic solutions, on the other hand, contained alcohol 30 per cent, the lowest percentage which could safely be relied on to destroy the pneumococci in sputum, and glycerin 10 per cent in addition to the isotonic quantities of sodium chloride and bicarbonate used in the bland solutions. Spirits of chloroform and oil of wintergreen were also used to disguise the taste of the alcohol. With these solutions the practical studies were conducted on pneumonia patients: the bland solution with a view of determining the effect of simple rinsing of the mouth, diluting and removing the secretions with the bacteria; the alcoholic solutions with a view of determining the value of disinfection. In health the pneumococci

are practically confined to the mouth or upper air passages. In pneumonia, virulent pneumococci are being continually coughed up from the lung. The pneumonia patient, however, sooner or later reaches a stage when living pneumococci are no longer coughed up from the lung. This stage is doubtless more quickly reached in those recovering by crises, and an attempt was made to secure some definite data on this point by a comparative study of the sputa from the mouth with that from the lungs or bronchi. Contaminations from the mouth, however, could not be excluded, so the results were not significant. In tuberculosis, clumps of exudate from the lung may be studied by Kitasato's method of repeated washing, but with the material obtained from pneumonia patients this was not practicable.

PRACTICAL TESTS OF MOUTH CLEANSING AND DISINFECTION IN PNEUMONIA.

Through the kindness of Dr. G. L. Peabody, cases of pneumonia at the Roosevelt Hospital, before and after the crisis and also during lysis, were given six ounces of the simple bland wash for rinsing the mouth and gargling the pharynx. For four days specimens of the secretion were collected in the morning before the mouth cleansing, after the mouth cleansing, and following this specimens of sputa were coughed up from the lungs and bronchi. Morphological examinations of these specimens were made but proved unsatisfactory and were shortly abandoned. Animal inoculation, however, proved quite sufficient. An eighth to a quarter of a cubic centimeter of the sputum was injected subcutaneously into mice. The results may be very briefly stated, for of these specimens, in all 40, none failed to kill mice by pneumococcus infection, and the animals died at regular and uniform intervals. It was thus definitely determined that rinsing and gargling the mouth and pharynx, as practiced in the hospital routine, is a useless procedure in so far as getting rid of the virulent pneumococci is concerned. Similar results were obtained in tests conducted on healthy individuals. Occasionally it was noted that an irritation of the mucous membrane followed by varying inflammatory conditions developed after this energetic mouth cleansing with the saline solutions.

Through the kindness of Dr. William H. Thomson, a series of

practical tests of the disinfecting action of the alcohol mouth wash* was conducted on cases of pneumonia at the Roosevelt Hospital. The tests were made in the same way as in the previous experiments, in which the bland saline solutions were used for the mouth cleansing. Larger quantities of sputum were used in the inoculations; each mouse received approximately 0.5 c.c. These results may also be briefly stated, for the specimens, 32 in all, never failed to show the presence of virulent pneumococci. The animals, however, in spite of the larger quantities of sputum inoculated, died at more irregular intervals than in the tests made with the bland saline solutions. In one case this was particularly marked. The two controls from this patient taken before the wash was used died in three and three and one-half days; subsequent controls in seven, eight, and four days; the tests in thirteen, eight, nine, and five days respectively. It was thus definitely established that disinfection of the mouth

*For convenience in the hospital practice the solution was so made up that the addition of water in equal parts gave the desired strength. The flavorings of spts. of chloroform and oil of wintergreen lose their strength after keeping a few weeks, so that it may be wise to guard against this by the substitution of other flavoring agents.

Sodium chloride	gr. xl
Sodium bicarbonate	" xx
Glycerin	"
Water (dist.)	aa 3 iss
Alcohol	3 v
Spts. of chloroform	3 ii
Oil of wintergreen	qts. x.

Dilute with water in equal parts.

Owing to the sweet taste and the evaporation of the chloroform and oil of wintergreen this solution has not proved as satisfactory in private practice as those containing other flavoring agents. Although it was found that the solution may be varied to suit the taste of the patient by the use of different flavoring agents, the following formula has given, in my experience, the best results:

		Metric
R Sodium chloride (C. P.)	3 ss	2.0 grams
Sodium bicarbonate (C. P.)	gr. x	0 7 "
Water (dist.)	3 ii	62.0 c.c.
Glycerin	3 i	31.0
Alcohol	3 v	155.0
Thymol	"	"
Menthol	aa gr. i	0.07 grams
Oil of wintergreen	gtt. iii	"
" " cinnamon	gtt. ii	"
" " eucalyptus	gtt. v	"
Tr. cut bear	3 iss	6.0 c.c.
Tr. rhatany	3 ss.	2.0

M. Sig. Dilute with water equal parts.

In preparing these solutions the salts should be dissolved in the water before adding alcohol. Even when carefully made up a cloudiness or a precipitate may appear in the solution, especially in those containing the tincture of rhatany. By adding two or three of the flavoring oils a less pronounced taste is obtained than when only one is used.

and pharynx by means of antiseptic mouth washes, as practiced in the hospital routine of pneumonia patients is incomplete.

Similar results were obtained in the practical tests conducted on healthy individuals, except in one instance where for 48 hours, so far as could be determined by animal inoculation, complete disinfection of the pneumococci in the secretion was secured with the alcohol solution. As compared with the experiments with the bland saline solutions, no irritation or inflammation of the mucous membrane followed the use of this alcoholic mouth wash.

Personal experience with the two methods, the simple and the antiseptic, yielded unexpected results. During the use of the bland salt solution pneumococci were always found by the inoculation of mice with the secretion. After the substitution of the alcohol antiseptic solution pneumococci, except occasionally at first, have not been obtained from the secretion under normal conditions. It thus appears that removing and disinfecting the contaminated secretions from the more accessible parts of the mouth with the alcohol wash may in time accomplish the elimination of pneumococci from the secretions of healthy persons.

By using an efficient antiseptic wash which is non-irritating, large numbers of virulent pneumococci, those present in the secretions and some of those remaining in the mouth, are destroyed, and the exposed mucous membrane is less apt to become infected; thus the danger, not only to the individual but to his fellows, from the infectious secretions, is materially lessened as compared with the cleansing with bland saline solutions having no antiseptic action. Furthermore, aside from its antiseptic action, the alcohol formula proved quite as bland and more effective in the mechanical cleansing of the mouth than the simple isotonic salt solutions; the secretions were more quickly removed from the mucous membranes and teeth; the irritation of simple alcohol solutions was relieved by the addition of glycerin and salts to such an extent that a mild beneficial stimulation, without injurious desiccation, was all that was experienced, and this was quickly followed by an increased flow of fresh secretion of comparatively pure mucus, which replaced the old contaminated saliva and sputa in the mouth and pharynx.

Thus it is that although complete disinfection of the mouth in

pneumonia is uncertain, the best results were secured by using for mouth cleansing an efficient antiseptic, such as alcohol, rendered bland and non-irritating.

SUMMARY AND CONCLUSIONS.

Although mouth cleansing has long been practiced, and although previous study of mouth disinfection has shown that complete disinfection is impossible and suggested many of the serious difficulties encountered in the general problem, the results of all this experience failed to determine accurately the best procedures or discriminate definitely between the efficient and the inefficient, even harmful, antiseptics. Similarly the results of the present studies show that complete disinfection of the mouth is impossible, but from the tests made in the various experiments the practical value of many of the antiseptic procedures commonly employed in mouth disinfection was accurately determined.

It was found that pneumococci, as compared with other pathogenic bacteria, are extremely susceptible to the plasmolytic action of saline and alkaline solutions and also to the antiseptics which act in this way. But the susceptibility to antiseptics in general varies greatly and especially under different conditions. In broth media the pneumococci are readily destroyed; in exudates the albuminous material and detritus interferes with the action of many antiseptics; in sputum, disinfection by harmless solutions is extremely difficult.

Of all the commercial solutions studied—listerine, borine, boro-lyptol, glycothymoline, odol, and Seiler's solution—none proved efficient when tested on pneumococci under the conditions most favorable for their action. Formalin, lysol, and hydrogen peroxide, failed to act on the pneumococcus in exudates. In short, alcohol alone of all the antiseptics studied proved efficient when tested on the pneumococci under all the conditions of the experiments.

Observations on the diffusion of the different solutions with the secretions of the mouth under different conditions showed that the presence of salts in isotonic quantities and alkalis was of positive, if slight, value. The rapid diffusion obtained with alcoholic solutions was greatly accelerated by the addition of glycerin. Finally, the results of these experiments suggested the use of hot solutions.

As shown by the practical studies, cleansing the mouth with simple isotonic salt solution removes a certain amount of the secretion and with it some of the infectious material, but does not destroy the bacteria. With the alcohol wash many of the bacteria are destroyed, the contaminated secretions are more rapidly removed and at the same time disinfected, and, finally, the natural resources of the tissues are more safely and efficiently aided in the elimination of the infectious material. It was thus determined that alcohol solutions containing glycerin and salts in bland quantities were in every particular more efficient than any of the washes hitherto recommended for mouth cleansing or disinfection; 30 per cent of alcohol being the strongest that can be comfortably and habitually used in the mouth and the weakest that will give reliable disinfection.

From the hygienic standpoint the secretions of the mouth constitute the chief, if not the only, source of respiratory infection and the infectious material is transformed from one person to another; in some instances through the air as from sneezing or coughing, but to a much larger and more serious extent, directly by personal contact or the use in common of the various accessories of life. In crowded communities and during seasons of the year when people are huddled closely together in narrow quarters, the vicious cycles by which the dissemination of the infectious material is fostered involve nearly every individual. Under such conditions the precautions and regulations within the jurisdiction of the health authorities, so successfully adopted with other infectious diseases, are obviously inadequate, and the responsible burden of alleviating or controlling the spread of the respiratory diseases rests largely upon the individual.

Whether it be for purposes of cleanliness or as a wise precaution in the care and preservation of the teeth, mouth cleansing of some sort will always be practiced. The present researches with reasonable precision show that not only is the simple cleansing best effected with bland alcoholic solutions, but as disinfectants in the mouth these solutions, of all those studied, alone possess practical value.

I wish, particularly, to express my indebtedness to Professor T. Mitchell Prudden for his suggestions and for his criticism.

BIBLIOGRAPHY.

1. ARLOING. *Jour. de physiol. et de path. gen.*, 1902, 4, p. 291.
2. BECO. *Archiv de med. expér.*, 1899, 11, p. 317; *ibid.*, 1901, 13, p. 51.

3. V. BESSER. *Zieglers Beiträge*, 1889, 6, p. 351-62.
4. BEZANCON ET GRIFFON. *Soc. med. des Hopitaux*, 1898, Apr. 15.
5. BONHOFF. *Centralbl. f. Bakt.*, 1902, 32, p. 641.
6. BÜRGER. *Jour. Exper. Med.*, 1905, 7, p. 545.
7. DANIELSHON AND HESS. *Deutsch. med. Wchnschr.*, 1902, 28, p. 662.
8. ENGELS. *Hand Disinjection*, 1905, G. Fischer, Jena.
9. FISCHER. *Zeitschr. f. Hyg.*, 1900, 33, p. 1.
10. FLECKSEDOR. *Centralbl. f. inn. Med.*, 1905, 26, p. 41.
11. FRAENKEL. *Zeitschr. f. klin. Med.*, 1886, 10, pp. 419, 420.
12. GILBERT ET CARNOT. *Compt. rend. de Soc. de biol.*, 1904, p. 925.
13. GREVE. *Prevention of Diseases of the Teeth and Mouth*, p. 769, Vol. II of *The Prevention of Disease*; translation published by Funk and Wagnalls, 1903.
14. HISS. *Jour. Exper. Med.*, 1905, 7, p. 581.
15. HOPKINS. *Internat. Dental Jour.*, 1903, Aug.
16. KOLLE U. WASSERMAN. *Handbuch der Pathogen Mikroorganismen*, 1903, 2, p. 779.
17. LEWANDOWSKY. *Archiv f. Hyg.*, 1904, 49, p. 47.
18. LONGCOPE AND FOX. *Jour. Exper. Med.*, 1905, 7, p. 430.
19. MILLER. *Dental Cosmos*, 1891, 33, p. 253.
20. MILLER. *Microorganisms of the Human Mouth*, 1890, p. 205.
21. MILLER. *Ibid.*, pp. 225-36; *Lehrbuch der Conservierenden Zahnheilkunde*, 1898, Leipzig, pp. 440-43.
22. MONIER. *Ref. Bull. l'Inst. Past.*, 1904, 2, p. 857.
23. MORISIANI. *Arch. di. ost. e. Ginec.*, 1897, 4, p. 133.
24. NETTER. *Soc. de biol.*, 1897, Nov. 20; *Traité de Méd.* (Bouchard et Brissaud) 1901, 6, pp. 455, 486.
25. NEUMANN. *Zeitschr. f. Hyg.*, 1902, 40, p. 33.
26. PARK. BIGGS, PARK, AND BEEBE, *Report on Bact. Investigation and Diag., of Diphtheria*, Health Dept., City of New York, 1895.
27. PARK AND WILLIAMS. *Jour. Exper. Med.*, 1905, 7, p. 407.
28. PELNAV. *Wien. klin. Rundschau*, 1902, pp. 197 and 222.
29. RITTER. *Weyl. Handbuch der Hygiene*, Supplement 2, 1901, "Zahn und Mund Hygiene," pp. 231 and 238.
30. RODELLA. *Centralbl. f. Bakt.*, 1904, Orig. 37, pp. 314-17.
31. ROSE. *Zeitschr. f. Hyg.*, 1901, 36, p. 161.
32. SCHAEFFER. *Therapeutische Monatshefte*, 1904, Nov.
33. STEFFEN. *Centralbl. f. Bakt.*, 1895, 18, p. 164; with Grawitz, *Berl. klin. Wchnschr.*, 1894, 31, p. 419.
34. STROGANOFF. *Centralbl. f. Gynäk.*, 1893, No. 40.
35. STURTZ. *Zeitschr. f. klin. Med.*, 1904, 52, pp. 422-69.
36. THOMSON AND HEWLETT. *Medical and Surgical Trans.*, 1895, p. 239; *Brit. Med. Jour.*, 1896, 1, p. 137.
37. WALTHARD. *Centralbl. f. Bakt.*, 1895, 17, p. 311; *Arch. f. Gynäk.*, 48, pp. 233-44.
38. WEIGL. *Archiv f. Hyg.*, 1902, 44, p. 273.
39. WIRGIN. *Zeitschr. f. Hyg.*, 1902, 40, p. 307; *ibid.*, 1904, 46, p. 149.
40. WOOD. *Jour. Exper. Med.*, 1905, 7, p. 621, 622.
41. WURTZ ET LEMOYEZ. *Compt. rend. de Soc. de biol.*, 1894.

CORRECTIONS.

P. 107, Table 5, 1st line: instead of "o.1," read "1.o."

P. 173, end of 1st line: insert "of."

P. 375, 9th line from bottom: instead of "fifty," read "five."

GENERAL INDEX

SUBJECT INDEX.

	PAGE
Adjuvant Action of Serum, Egg-Albumin, and Broth on Tetanus Intoxi- cation - - - - -	116
Amanita Phalloides, Toxins and Antitoxins of - - - - -	191
Anthracidal Action of Dog Blood - - - - -	102
Anthracidal Substance in Serum of White Rats - - - - -	110
Antipneumococcal Power of the Blood in Pneumonia - - - - -	731
Antitoxins, Oral Administration of - - - - -	701
Are Opsonins Distinct from Other Antibodies? - - - - -	434
Auxylins - - - - -	225
Bacilli, Diphtheria, Phagocytosis of - - - - -	441
Bacilli, Diphtheria, Method of Staining Polar Bodies of - - - - -	770
Bacilli pseudodiphtheriticus - - - - -	128
Bacillus aerogenes var. lacticus - - - - -	181
Bacillus coli - - - - -	547
Bacillus diphtheriae - - - - -	128
Bacillus hemophilicus - - - - -	34
Bacillus influenzae - - - - -	34
Bacillus pertussis - - - - -	34
Bacillus typhosus, Toxic Effect of Acids upon - - - - -	547
Bacteriology of Whooping-Cough - - - - -	1
Blastomycosis dermatitidis - - - - -	679
Blastomycosis, Disseminated - - - - -	91
Blastomycosis, Generalized - - - - -	666
Coccaceae, Statistical Study of Generic Characters in - - - - -	485
Colonies in Blood-Agar Plates - - - - -	663
Contribution to the General Principles of the Pharmacodynamics of Salts and Drugs - - - - -	572
Cytotoxic Sera, Production of, by Nucleoproteids - - - - -	742
Diphtheria and Tetanus Toxins, Horses Immunized with - - - - -	619
Drugs and Salts, Pharmacodynamics of - - - - -	572
Drugs, Influence of, in Digestion and Absorption - - - - -	701
Filaria loa - - - - -	37
Fundamental Error in Attempts to Apply Physical Chemistry to Serum Pathology - - - - -	638
Further Studies on Streptococcus Infections - - - - -	136
Further Studies on Virulent Pseudodiphtheria Bacilli - - - - -	128

	PAGE
Fusiform Bacilli and Spirilla, Identity of - - - - -	148
Generic Characters in the Coccaceae - - - - -	485
Green Coloration of Bacterial Colonies on Blood-Agar Plates - - -	663
Human Parasites - - - - -	37
Hydrophobia, The Etiology and Diagnosis of - - - - -	452
Influenza-like Organisms - - - - -	14, 25
Latent Pneumococchemia - - - - -	446
Liver Necrosis and Venous Thrombosis in Horses - - - - -	619
Method of Isolating the Pneumococcus - - - - -	183
Method of Staining Encapsulated Pneumococci - - - - -	610
Method of Staining the Polar Bodies of Diphtheria Bacilli - - -	770
Method of Sterilizing Blood for Cultural Purposes - - - - -	772
Methylene Violet and Methylene Azure - - - - -	412
Micrococcus catarrhalis - - - - -	22, 26, 30
Micrococcus pemphigi contagiosi - - - - -	171
Mosquitoes of the Bihé and Bailundo Districts - - - - -	187
Mouth Disinfection in Pneumonia - - - - -	774
Mushrooms, Poisonous - - - - -	191
Negri Bodies - - - - -	463
Nucleoproteids, Production of Cytotoxic Sera by - - - - -	742
Oidiomycosis - - - - -	666
Oposonin for Anthrax Bacilli in Dog Serum - - - - -	102
Oposonic Index and the Antipneumococcal Power of the Blood - - -	731
Oposonins, Are They Distinct from Other Antibodies? - - - - -	434
Oral Administration of Antitoxins - - - - -	701
Pemphigus contagiosus in the Tropics, The Etiology of - - - - -	165
Pertussis - - - - -	1
Phagocytosis - - - - -	102
Phagocytosis of Diphtheria Bacilli - - - - -	441
Phagocytosis of Red Corpuscles - - - - -	721
Phagocytosis, Rôle of, in Pneumococcal Action of Pneumonic Blood -	683
Pharmacodynamics of Salts and Drugs - - - - -	572
Physical Chemistry and Serum Pathology - - - - -	648
Pneumococchemia, Latent - - - - -	446
Pneumococci, Simple and Differential Methods of Staining - - -	610
Pneumococcus, A Method of Isolating the - - - - -	183
Pneumonia, The Blood in - - - - -	731
Pseudodiphtheria Bacilli, Further Studies on - - - - -	128

	PAGE
Relapsing Fever, Two Cases of - - - - -	233
Serum Component, The Third - - - - -	647
Serum Pathology - - - - -	638
Spirillum Duttoni - - - - -	380
Spirillum glossinae, n. sp. - - - - -	385
Spirillum Obermeieri and Related Organisms - - - - -	291
Spirochete from a Case of Relapsing Fever - - - - -	266
Streptococci from Scarlatinal and Normal Throats - - - - -	755
Streptococci, Horses Immunized with - - - - -	619
Streptococci in Milk, The Significance of - - - - -	173
Streptococcus lacticus - - - - -	181
Streptococcus pyogenes, Coloration of Colonies - - - - -	663
Tetanus Intoxication, Adjuvant Action of Serum, Broth, and Egg-Albu- min on - - - - -	116
Tetanus Toxins, Horses Immunized with - - - - -	619
Tick Fever - - - - -	379
Toxic Effects of Certain Acids upon Typhoid and Colon Bacilli - - - - -	547
Toxins and Antitoxins of Poisonous Mushrooms - - - - -	191
Trypanosoma Grayi, n. sp. - - - - -	405
Whooping-Cough, The Bacteriology of - - - - -	1
Venous Thrombosis in Horses - - - - -	619

INDEX OF AUTHORS.

	PAGE
BASSOE, PETER - - - - -	91
BERNSTEIN, EUGENE P. (and EPSTEIN, ALBERT A.) - - - -	772
CARLISLE, ROBERT J. - - - - -	233
CLEGG, MOSES E. (and WHERRY, WILLIAM B.) - - - -	165
DAVIS, DAVID J. - - - - -	1
EPSTEIN, ALBERT A. - - - - -	770
EPSTEIN, ALBERT A. (and BERNSTEIN, EUGENE P.) - - - -	772
FLOURNOY, THOMAS (NORRIS, CHARLES, and PAPPENHEIMER, ALVIN M.)	266
FORD, WILLIAM W. - - - - -	191
GRAHAM, E. A. (and IRONS, E. E.) - - - - -	666
HAMILTON, ALICE (and HORTON, JESSIE M.) - - - - -	128
HEINEMANN, PAUL G. - - - - -	173
HEKTOEN, LUDVIG - - - - -	102, 434, 721
HORTON, JESSIE M. - - - - -	110
HORTON, JESSIE M. (and HAMILTON, ALICE) - - - - -	128
IRONS, E. E. (and GRAHAM, E. A.) - - - - -	666
JACKSON, HOLMES C. (and PEARCE, RICHARD M.) - - - -	742
KENDALL, ARTHUR I. - - - - -	228
KING, WALTER E. (and MCCLINTOCK, CHARLES T.) - - - -	701
KIRK, E. J. (and RICKETTS, H. T.) - - - - -	116
KNAPP, R. E. (and NOVY, FREDERICK G.) - - - - -	291
LOCHRIDGE, E. E. (and WINSLOW, C. E. A.) - - - - -	547
LOWDEN, MAY M. (and WILLIAMS, ANNA W.) - - - - -	452
MACNEAL, WARD J. - - - - -	412
MANWARING, WILFRED H. - - - - -	225, 638, 647
MATHEWS, A. P. - - - - -	572
MCCLINTOCK, CHARLES T. (and KING, WALTER E.) - - - -	701
NORRIS, CHARLES (PAPPENHEIMER, ALVIN M. and FLOURNOY, THOMAS)	266
NOVY, FREDERICK G. - - - - -	394
NOVY, FREDERICK G. (and KNAPP, R. E.) - - - - -	291

	PAGE
PAPPENHEIMER, ALVIN M. (NORRIS, CHARLES, and FLOURNOY, THOMAS)	266
PEARCE, RICHARD M. (and PEASE, HERBERT) - - - - -	619
PEARCE, RICHARD M. (and JACKSON, HOLMES C.) - - - - -	742
PEASE, HERBERT D. (and PEARCE, RICHARD M.) - - - - -	619
REQUE, H. A. - - - - -	441
RICKETTS, H. T. (and KIRK, E. J.) - - - - -	116
ROGERS, ANNE F. (and WINSLOW, C. E. A.) - - - - -	485
ROSENOW, EDWARD C. - - - - -	683
RUEDIGER, GUSTAV F. - - - - -	156, 183, 663, 755
TUNNICLIFF, RUTH - - - - -	148
WADSWORTH, AUGUSTUS - - - - -	610, 774
WARD, HENRY B. - - - - -	37
WELLMAN, FREDK. CREIGHTON - - - - -	187
WHERRY, WILLIAM B. (and CLEGG, MOSES E.) - - - - -	165
WILLIAMS, ANNA W. (and LOWDEN, MAY M.) - - - - -	452
WINSLOW, C. E. A. (and LOCHRIDGE, E. E.) - - - - -	547
WINSLOW, C. E. A. (and ROGERS, ANNE F.) - - - - -	485
WOLF, HERMAN E. - - - - -	446, 731

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